GROWTH

A JOURNAL FOR STUDIES OF DEVELOPMENT AND INCREASE

VOLUME XXIV
PAPERS 578-597

1960

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84
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vol. 24
no. 1

GROWTH

A JOURNAL FOR STUDIES OF DEVELOPMENT AND INCREASE

THE UNIVERSITY OF MICHIGAN
JUN 21 1960
SCIENCE LIBRARY

VOLUME XXIV, NUMBER 1
PAPERS 578-582

MARCH, 1960 $6.00 A YEAR

Second-Class postage paid at Worcester, Mass.

GROWTH

A journal for the coordination of studies of increase and development as general properties of nature. Published by contributors and subscribers on a cooperative, non-profit, non-salary basis. The field of investigation is primary and work will be published in which the approach is through mathematics, physics, chemistry, genetics, embryology, philosophy, and anthropology; just as will studies of development in its several expressions, and of mass increase and the part played therein by nutrition, vitamins, and hormones.

It is published four times a year during March, June, September and December at $6.00 per volume (outside U. S. and Canada, $6.50). Single numbers $2.00. There will be one volume each year.

All manuscripts and subscriptions should be sent to: The Editors of Growth, The Institute for Cancer Research, Fox Chase, Philadelphia 11, Pa.

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Growth, 1960, **24**, 1-27

EXPERIMENTAL STUDIES ON MITOMYCIN C. 2. CYTOTOXICITY*

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Received February 18, 1960

In our previous paper, "Experimental Studies on Mitomycin C. 1." published in this journal (43), we pointed out that Mitomycin C has a tendency to accumulate in tissues of the liver and the spleen, and to a lesser degree in the kidneys and other organs. Due to this accumulation of the antibiotic, it was suggested that intermittent rather than consecutive application might be advantageous, thus minimizing the toxic side effects of this therapy. The clinical trials conducted by Miller and McLemore (27) and Shimada *et al.* (41), and the results obtained by them, confirmed our experimental data in this respect.

The chief features of Mitomycin C cytotoxicity in animals, rats, mice and chicks were loss of weight, leucopenia, internal hemorrhage, and a mild neutropenia according to Sokoloff *et al.* (43). These investigators also reported that baby chicks given Mitomycin C occasionally showed alopecia. Sternberg *et al.* (46) observed that Mitomycin C affected hematopoietic tissues and intestinal epithelium in rats, mice and dogs. It induced a necrotizing nephrosis and hemorrhagic diathesis in Rhesus monkeys. While the Japanese workers, Shiba *et al.* (40); Sukie *et al.* (47); Shimada *et al.* (42) and others were inclined to minimize the gravity of the side-effects in the clinical administration of Mitomycin C, Colsky *et al.* (8), reported leucopenia, neutropenia, hemorrhagic diathesis, diarrhea, anorexia and occasional alopecia, when the consecutive method of Mitomycin therapy was applied.

* Aided by grants from the National Cancer Institute, CY-4757(A), the Damon Runyon Memorial Fund for Cancer Research, DRG-485, and the Marcia Tucker Foundation, N. Y.

Fully cognizant of a relatively high cytotoxicity of Mitomycin C, we have attempted in the present study to reduce it by various physiological "protective measures," such as bone marrow cell transplantation, spleen extracts, administration of certain anti-inflammatory factors and the H-S amino acids, as well as activation of the Reticulo-Endothelial-System.

Bone Marrow Cell Transplantation

The extensive experimental studies of recent years on the recovery of bone marrow from the injury caused by exposure to a total-body ionizing radiation by intravenous transplantation of living bone marrow cells demonstrated the usefulness of this method. The work of Congdon and Urso (9); Porter and Murray (31); Ferrebee *et al.* (12); Porter (30); Rekers *et al.* (35); Barnes *et al.* (4); Barnes and Loutit (5) and others showed that by infusion of marrow cells, the fatal outcome from irradiation was prevented in mice, rats, dogs and various animals. Crouch and Overman (10) found that homologous bone marrow would protect 60 per cent of the Rhesus monkeys given 600 r. Rothberg *et al.* (36) confirmed their observation and, in turn, reported that homologous marrow transplantation gave some protection to irradiated monkeys when the dose did not exceed 900 r. The attempt to apply this method in clinical practice met however, with numerous obstacles. Thomas *et al.* (48) gave the infusion of bone marrow cells to ten patients affected with acute leukemia and receiving total body irradiations and to one case of leukemia receiving a massive dose of nitrogen mustard. Only two cases may be said to have obtained significant but not impressive objective benefit. Similar results were obtained by McFarland *et al.* (21) in five cases of malignant diseases treated with large doses of nitrogen mustard and given autologous bone marrow infusion. Two of the five patients succumbed to infection, with the other three having only a brief remission. Some more encouraging results were reported at the Bone Marrow Transplantation Conference (7).

However, when an improved technique of bone marrow transplantation in man, described recently by Schwartz, Misra, and Dameshek (39) was applied, quite encouraging results were obtained in one hundred cases.

Weston (53) studying Myleran, an oncostatic drug of high toxicity,

demonstrated that a single 20 mg. per kg. dose of this agent, given intravenously to rats, resulted in a severe hypoplasia of the marrow, with the total nucleated cell count going down to 250,000 cells per cu. mm. The mortality rate among the rats was as high as 90 per cent. The injury to the bone marrow was permanent and there was no evidence that a Myleran-poisoned marrow attempted to come back in two weeks or more. Myleran also induced neutropenia according to Pujman (32).

Weston (53) used bone marrow transplantation successfully for minimizing severity of the bone marrow injury induced by Myleran. The intravenous injection of 125 million nucleated rat cells resulted at day 14 after Myleran poisoning in about a 70 per cent return of the marrow to a normal active state.

In our present experiments, we followed closely the technique of bone marrow transplantation used by Weston. The bone marrow was obtained from femurs and humeri of rats of the same litter, scraped out with a microscopic curet and pushed through a 30-mesh stainless screen into Ringer's solution at pH 7.3. Nucleated cell counts were made by conventional system. All this procedure was conducted under sterile conditions. From 75 up to 120 million nucleated rat marrow cells, 0.5 ml., were inoculated intravenously to rats given heavy doses of Mitomycin C. The inoculation was administered after the rat received three injections of the antibiotic. In the first series of tests, fifteen rats were given a 3.0 mg. per kg. wt. dose of Mitomycin C for seven consecutive days. Such a dose was very toxic and many rats succumbed after the fourth or fifth injection of Mitomycin C. The rats which survived seven or more days showed strong leucopenia, with the white blood cell number going down to about 1,500. The mortality rate in this series was 87 per cent. Another fifteen rats, receiving the same dose of Mitomycin C, were given intravenously, 75 million nucleated marrow cells. The mortality rate in this group was also high, but less so than in the Mitomycin C group: 40.2 per cent. Leucopenia was less pronounced, although the number of white blood cells had dropped down considerably, as compared with the normal rats.

More satisfactory results were obtained when the Mitomycin C daily dose was smaller and the number of nucleated cells inoculated to rats larger. Table I summarizes the results of this series of tests.

TABLE I

EFFECT OF HOMOLOGOUS RAT MARROW CELL TRANSPLANTATION ON THE TOXICITY OF MITOMYCIN C

Group A. 15 albino rats, males, given 2.0 mg./kg. wt./rat/day, every second day, seven injections.

Group B. 15 albino rats, males, given 2.0 mg./kg. wt./rat/day, every second day, seven injections. After the third injection of Mitomycin C, 125 million nucleated rat cells were inoculated intravenously.

Groups	Initial Wt. av. gm.	Final wt. 15 days av. gm.*	WBC on 15th day*	Mortality Rate %
Group A				
15 rats Mitomycin C total dose 14 mg./kg. wt./rat given in 14 days	149.3	122.3	2,320	40
Group B				
15 rats Mitomycin C total dose 14 mg./kg. wt./rat given in 14 days and 125 million nucleated rat cells inoculated on seventh day	151.1	143.5	4,650	13.3

* Only the survived rats are included in this count.

The mortality rate in the Mitomycin C group was still high: 40 per cent, with the surviving rats showing leucopenia. The mortality rate in the group receiving bone marrow transplantation of 125 million cells was 13.3 per cent with no evidence of leucopenia, but with the number of white blood cells somewhat below the normal level. It should be stated that in spite of a decreased mortality rate and a higher white blood cell count in this group of rats, the animals lost considerable weight.

Comment: The results of our experiments with the homologous marrow cell transplantation to rats receiving toxic doses of Mitomycin C indicate that this method prevented to some degree bone marrow depression and decreased mortality rate of the treated animals.

SPLENECTOMY AND SPLEEN EXTRACTS

Ultmann (49) demonstrated that there was a splenic overactivity and certain dysfunction in cancer patients, the nature of which he

has not defined. He assumed that hemolysis seemed to be a contributing factor to the anemia of malignant diseases. White (54) on the basis of his experimentation with nitrogen mustard on splenectomized dogs concluded that the toxicity of this compound was at least partly related to the formation within the spleen of some toxic materials. Sokoloff *et al.* (43) found that administration of Mitomycin C to rats and chicks resulted in shrinkage of the spleen, the same phenomenon as observed by them in the case of nitrogen mustard. Accordingly, we have investigated the effect of splenectomy and of spleen extracts on cytotoxicity of Mitomycin C.

a. *Splenectomy.* Twenty white mice, all males, average weight 22.5 gm., were splenectomized. Six days after the operation, they were divided into two groups of ten mice each. Group A served as control. Group B, received Mitomycin C, 0.06 mg/mouse/day for seven consecutive days, intraperitoneally. Group C, normal mice which were not splenectomized, were also given Mitomycin C, 0.06 mg/mouse/day for seven consecutive days. The following results were obtained on the eighth day after termination of the experiment.

Group A. Control, 10 mice. Splenectomized. All alive.

Group B. 10 splenectomized mice, receiving Mitomycin C, a total dose 0.42 mg/mouse, in seven days. 5 mice dead, 5 alive.

Group C. 10 normal, not splenectomized mice, receiving Mitomycin C, a total dose 0.42 mg/mouse, in seven days. 4 mice dead, 6 alive.

These experiments with toxic doses of Mitomycin C were repeated on mice bearing Ehrlich carcinoma, with similar end-results.

Comment: Splenectomy did not decrease the cytotoxicity of Mitomycin C administered to mice, both normal or cancerous.

b. *Spleen extracts.* Savitsky (37) described a procedure of preparing a spleen extract fraction, containing the so-called TCRA, or the anti-hemorrhagic factor. Following his method, the Wilson Laboratories, Chicago, supplied us with such a spleen extract.

Forty-five mice, all males, average weight 24.0 gm. were divided into three groups of fifteen mice each. Group A, control, receiving only spleen extract TCRA, 0.1 mg/0.1 ml. intraperitoneally for seven days. Group B was given Mitomycin C, 0.05 mg/0.1 ml./mouse/day with 0.1 mg. spleen extract, mixed together, for seven consecutive days.

Group C, Mitomycin C alone, 0.05 mg/mouse/day for seven consecutive days. Seven days after termination of the experiment, it was found that the group receiving Mitomycin C with spleen extract showed a moderate decrease in the toxic side effects of therapy.

Group A. Only spleen extract TCRA. All mice alive. Average weight gain: 1.1 gm.

Group B. Mitomycin C and spleen extract TCRA. 1 mouse dead; 14 alive. Average weight gain: 0.15 gm.

Group C. Mitomycin C alone. 3 mice dead; 12 alive. Average weight loss: 1.3 gm.

Comment: The spleen extract, containing the anti-hemorrhagic factor TCRA administered to mice receiving toxic doses of Mitomycin C, moderately decreased the side effects of this therapy, with the mortality rate smaller in the spleen extract-treated group, than in the control group receiving Mitomycin C alone.

Local Skin Inflammatory Reaction

Mitomycin C, injected intradermally to a rabbit abdomen, in a 0.05 or 0.075 mg. dose, caused an acute inflammatory skin reaction. A smaller dose, 0.02 mg., induced a milder reaction. Figure 1 presents typical reactions induced by three doses, 0.02 mg., 0.05 mg., and 0.075 mg. of Mitomycin C. When 6.0 ml. of a 1 per cent solution of trypan blue is inoculated intravenously, 24 hours after Mitomycin C intradermal injection, there is a clear accumulation of blue dye in the inflamed areas (Figure 2). The histological examination of these areas of the skin shows a typical inflammatory process with leucocytic migration (Figure 3). Twenty-four hours after trypan blue inoculation, the skin areas injected with Mitomycin C showed a change in their appearance. The central portion of the areas no longer had dye accumulation, which remained on the periphery. It underwent a necrotizing process (Figure 4).

Menkin (22) reported that the inflammatory reaction and leucocytic migration, induced by intradermal injections of an inflammatory exudate or leukotaxine, was prevented by an anti-inflammatory factor of a flavonoid nature. The dye accumulation, indicative of increased capillary permeability, was absent in rabbit abdomen skin when 0.25 ml. inflammatory exudate was injected together with water-

FIGURE 1

Mitomycin C, 0.02, 0.05 and 0.075 mg. was injected intradermally. Several hours after, inflammation is visible in the injected areas.

Diameter of inflammation areas:

2.0	4.0	5.5 cm.
5.5	5.5	2.5 cm.

soluble flavonoids at the pH 7. Employing the same flavonoid material as Menkin did, in our tests with Mitomycin C, we found that flavonoids, known for their capillary permeability activity, prevented or almost prevented appearance of the inflammatory reaction and of dye accumulation when injected, intradermally, together with Mitomycin C. Figure 5 illustrates these findings.

A rabbit, 3200 gm. weight, was injected intravenously with 1 per cent trypan blue solution, 6.0 ml. 24 hours prior, Mitomycin C, 0.05 mg/0.1 ml. was injected intradermally in four areas of the skin as follows:

O

O

O

O

FIGURE 2

Trypan blue accumulation in the skin areas, injected with Mitomycin C, 0.02, 0.05 and 0.075 mg. The diameters of the areas:

3.2	4.0	5.7 cm.
2.3	3.8	5.8 cm.

The same dose of Mitomycin C, 0.05 mg/0.1 ml. wt. plus 5 mg. of water-soluble flavonoids was injected in four areas as follows:

O

O

O

O

Figure 5 shows a strong accumulation of the dye in the first four areas, and none, or a smaller accumulation in the last four areas. The histologic examination of the areas injected with Mitomycin C and the anti-inflammatory factor indicates almost normal skin appearance. The anti-inflammatory factor injected intramuscularly and separately from Mitomycin C has no effect on the inflammatory reaction. The combination of Mitomycin C and the anti-inflammatory factor did not affect the anti-tumor activity of the antibiotic when tested on mice, bearers of Ehrlich carcinoma.

FIGURE 3

The skin area injected intradermally with Mitomycin C, 0.05 mg., showing an inflammatory process and leucocytic emigration. No hemorrhage is visible. x 100. Hematoxylin-eosin.

FIGURE 4

Twenty-four hours after trypan blue was injected intravenously to a rabbit, with Mitomycin C, 0.02, 0.05 and 0.075 mg. injected intradermally. The dye is accumulated only on the periphery of the areas, the central portions of which are necrotized.

Comment: Mitomycin C, injected intradermally to a rabbit abdomen in 0.05 mg. dose or higher induced an acute local inflammatory reaction. This reaction was absent, or considerably minimized when the anti-inflammatory factor, described by Menkin, was combined with Mitomycin C.

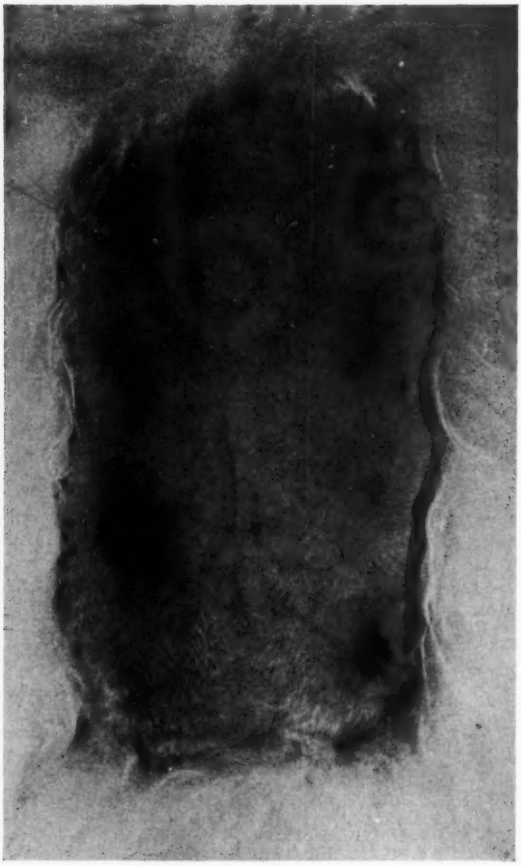

FIGURE 5

Anti-inflammatory activity of flavonoids. Four areas are injected with Mitomycin C, 0.05 mg. each about 5 cm. in diameter dye accumulation. Four areas are injected with Mitomycin C, 0.05 mg. and 5 mg. flavonoids. The injected areas are either absent from dye or of a diameter, 1.5 cm.

Activation and Blockade of the Reticulo-Endothelial-System

The intrinsic nature of Mitomycin C cytotoxicity is unknown, as is that of some other oncostatic antibiotics. In our study, an attempt was made to investigate the influence, if any, on toxicity of Mitomycin C of activating and blockading the R.E.S. We took as the departure point for our comparative experiments, the assumption that there exists an interaction between bacterial endotoxins and the R.E.S.

For "blockading" the R.E.S. we used the colloidal carbon preparation in fish glue, made by Gunther-Wagner, Hanover, Germany, C-11-1431A, 2.5 mg./100 gm. wt., and proferrin, saccharated iron oxide (Merck Co.) 2.5 mg/100 gm. wt.

For activation of the R.E.S., both hematoporphyrin chloride recrystallized, 2.5 mg./100 gm. wt. and Congo red, 2.0 mg./100 gm. wt. were employed.

a. *Blockade of the R.E.S.* We employed the liposaccharide prepared from *E. coli* by the Merck Institute for Therapeutic Research. We would like to thank Dr. George E. Boxer of the Institute for the generous supply both of the liposaccharide and proferrin.

When the colloidal carbon was injected intravenously to a rabbit, it was found that two or three hours thereafter the carbon particles were fixed in the phagocytic cells of the liver and spleen. When, however, the injection of colloidal carbon was preceded by the injection of *E. coli* liposaccharide, (3 mg./kg. wt.), the carbon particles did not appear in the phagocytic cells of the liver, but were detected in the endothelium of the small blood vessels and particularly within the kidney glomerular capillaries.

If, instead of the endotoxin, Mitomycin C (1.0 mg./kilo wt.) was injected to a rabbit, a similar deposition of carbon particles was observed, but it was much less pronounced. Some particles still were fixed in the phagocytic cells, while some others were observed in the glomerular capillaries. Increase of the dose of Mitomycin C, up to 2 mg./kilo wt., did not modify the type of reaction.

A similar observation was made with saccharated iron oxide, and local skin reaction. The endotoxin injected intradermally to a rabbit abdomen induced a local inflammatory reaction, the acuteness of which dependend on the dosage. When, however, saccharate iron oxide was injected intravenously, 15 mg./kilo wt., prior to the endotoxin injection, the skin reaction was absent or almost absent for about 15

hours. At about that time, numerous petechiae appeared in the injected areas, gradually forming a large hemorrhage, indicative of a Schwartzman-like phenomenon.

If, on the other hand, instead of endotoxin of 0.05 mg. or 0.1 mg., Mitomycin C was injected intradermally, preceded by intravenous injection of saccharated iron oxide (15 mg./kilo wt.), there was a certain similarity in the skin reactions. Soon after the injection there was slight reddening of the areas, which 12-15 hours thereafter showed multiple small hemorrhages, mostly around the small blood vessels and capillaries of the skin. Figures 6 and 7 show the skin

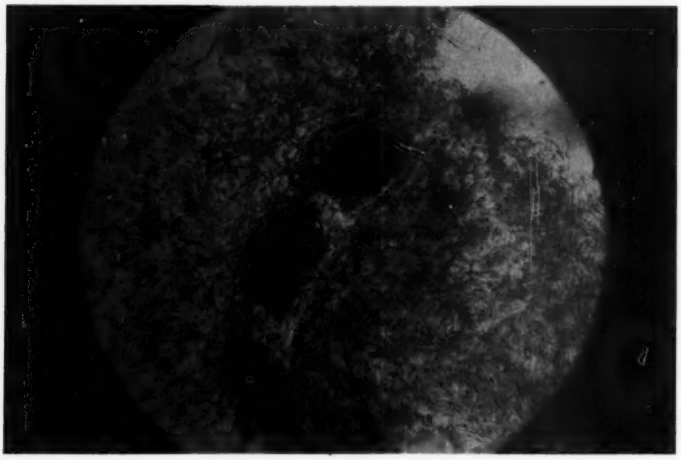

FIGURE 6

Rabbit skin. Blockade of the R.E.S. with saccharate iron oxide, 25 mg./kilo wt. Mitomycin C 0.05 mg. injected intradermally. 24 hours after injection, multiple small hemorrhages instead of inflammatory process. The tissue is filled with red blood cells. Hematoxylin-eosin. x 100.

areas injected with Mitomycin C with the R.E.S. blockaded by saccharated iron oxide. There was no evidence of inflammation and leucocytic migration, as was the case of the rabbit skin injected with the same dose of Mitomycin C, but without blockading the R.E.S. (Figure 3).

Comment: There is a certain similarity between the skin reactions

induced by *E. coli* liposaccharide and by Mitomycin C, when the R.E.S. is blockaded by saccharated iron oxide. However, no typical Schwartzman-like phenomenon was observed with Mitomycin C, either in these tests or on rats with massive doses of Mitomycin C injected intraperitoneally.

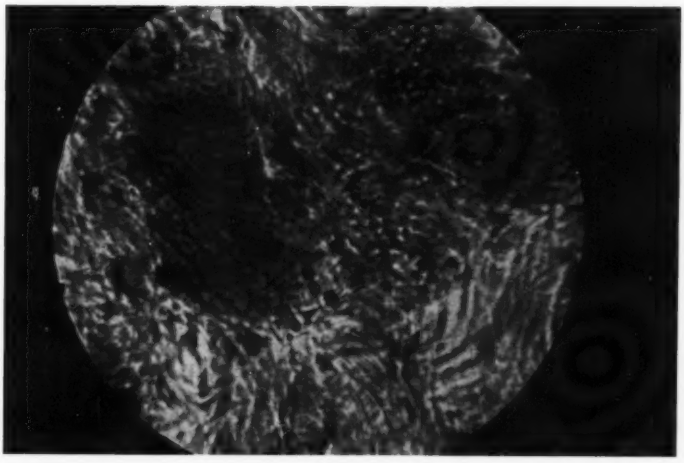

FIGURE 7
The same slide as Figure 6, showing hemorrhage. Hematoxylin-eosin. x 440.

Effect of Blockade of the R.E.S. on Leucopenia

We used rats for this series of experiments. Mitomycin C, (2.0 mg./kg. wt./rat/day), was administered intraperitoneally, every second day, a total of six injections. Two hours prior to Mitomycin C injection, saccharated iron oxide, (2.5 mg./100 gm. wt.) was injected intravenously. The results of these tests are summarized in Table II.

From this table, one may see that blockade of the R.E.S., prior to Mitomycin C administration, somewhat accentuated the toxic manifestations of this treatment. Weight loss was slightly increased. The mortality rate went up, from 33.3 per cent in the Mitomycin C group, without blockading, to 53.3 per cent in the group of rats, given both saccharated iron oxide and Mitomycin C.

The most interesting feature of blockading the R.E.S. was a further

TABLE II

EFFECT OF SACCHARATE IRON OXIDE ON THE TOXICITY OF MITOMYCIN C

Group A. 15 albino rats, males, given 2.0 mg./kg. wt./rat/day, every second day, six injections.

Group B. 15 albino rats, males, given 2.0 mg./kg. wt./rat/day, every second day, six injections, each injection preceded two hours prior by an intravenous injection of saccharate iron oxide, 2.5 mg./100 gm. wt./rat.

Groups	Initial Wt. av. gm.	Final wt. 13th day av. gm.*	WBC on 13th day av.*	Mortality Rate %
Group A				
15 rats Mitomycin C total dose 12 mg./kg. wt./rat given in 12 days	136.5	118.8	3,420	33.3
Group B				
15 rats Mitomycin C total dose 12 mg./kg. wt./rat given in 12 days and saccharate iron oxide, total dose 15 mg./rat given in 12 days	138.4	108.6	2,150	53.3

* Only the survived rats are included in the tabulation.

decline in the number of white blood cells in rats receiving near-toxic doses of Mitomycin C as compared with rats given only Mitomycin C. There was also an increased tendency to neutropenia in rats with the R.E.S. blockaded.

Comment: There is an indication that blockade of the R.E.S., in rats receiving Mitomycin C., aggravated the toxic side effects induced by the antibiotic.

b. *Activation of the Reticulo-Endothelial System.* Two compounds were used for activating the R.E.S.: hematoporphyrin and Congo red.

1. *Hematoporphyrin.* Auler and Banzer (3) were the first to point out that hematoporphyrin tends to accumulate in neoplastic, actively multiplied, tissue. Since hematoporphyrin exhibits a typical bright red fluorescence under near-ultra-violet light, it was suggested that this substance be used for detection of cancer. Figge *et al.* (13) and

Manganiello and Figge (19) have demonstrated accumulation of hematoporphyrin in traumatic and inflamed tissue, particularly with mitosis present. Peck *et al.* (28) showed the affinity of this substance to lymphatic tissue. Meyer-Betz (27) reported deposition of hematoporphyrin in the biliary duct and Peck *et al.* (29) suggested its possible use in surgery of the gall bladder. Figge (14) and Rasmussen-Taxdal *et al.* (33) used hematoporphyrin in surgery of the liver and bilary duct. They found this substance non-toxic even if injected in a dose of 1.0 gm. to their patients. By applying hematoporphyrin in surgery of cancerous growth, Rasmussen-Taxdal *et al.* (34) demonstrated that a large dose of this compound, up to 1.0 gm., should be used for proper accumulation of it in cancerous tissue. Della-Volpe and Albertini (11) reported that hematoporphyrin increased the resistance of rats to Walker adenocarcinoma. According to Weissberg (52) hematoporphyrin is not bound either chemically or by absorption to the tumor cells, nor is it phagocytized by these cells. It is accumulated in malignant tissue mostly within the foci of inflammation or in the case of congestive oedema.

Investigating hematoporphyrin and its accumulation in the tissue of animals, we found that shortly after intravenous injection, this substance was deposited chiefly in the liver, as revealed by fluorescence. The histologic examination indicated that hematoporphyrin was found mostly in histiocytes. Injected intravenously, it was detected within lymph vessels, only in the case of resorption of an inflammatory exudate. It was not found in lymph vessels of normal rats. Seven to ten hours after intravenous injection, hematoporphyrin was gradually eliminated from the liver and a large part of it was observed in the descending colon and rectum. About one per cent of hematoporphyrin was deposited in the skin, which fact necessitated keeping the patients out of sun rays, as stressed by Rasmussen-Taxdal *et al.* (34). The intravenous or intraperitoneal injection of hematoporphyrin to rats and mice resulted, according to our observation, in reticulocytosis of short duration, leucocytosis and neutrophilia.

According to our tests, hematoporphyrin, injected prior to Mitomycin C, prevented leucopenia in rats. Table III presents the results of these tests.

The rats of Group A, receiving Mitomycin C, 2.0 mg./kg. wt./rat/day, every second day, six injections, manifested leucopenia. The rats

TABLE III

Group A. 15 Albino rats, males, given 2.0 mg./kg. wt./rat/day, every second day, six injections.

Group B. 15 albino rats, males, given 2.0 mg./kg. wt./rat/day, every second day, six injections preceded by two hours with intravenous injections of hematoporphyrin, 2.5 mg./100 gm./rat.

Groups	Initial Wt. av. gm.	Final wt. 8th day av. gm.*	WBC initial av.	WBC final 8th day av.*	Mortality Rate %
Group A					
15 rats Mitomycin C total dose 12 mg./kg. wt./ rat in 12 days	121.5	102.3	7,850	2,650	40
Group B					
15 rats Mitomycin C total dose 12 mg./kg. wt./ rat in 12 days and hematoporphyrin, 7.5 mg./ rat in 12 days	118.9	106.2	7,930	10,230	13.3

* Only the survived rats are included in the tabulation.

of Group B, given the same dose of Mitomycin C with hematoporphyrin, 2.5 mg./100 gm. wt./rat/day, injected two hours prior to the Mitomycin C administration, showed leucocytosis, which was present in spite of Mitomycin C leucopenic influence. There was an average 17 per cent neutrophilic cells in the rats of Group A, and an average 27 per cent neutrophils in Group B. Thus hematoporphyrin seemed to prevent neutropenia induced by Mitomycin C in rats.

There is an indication that hematoporphyrin enhances moderately the oncostatic activity of Mitomycin C. In these series of tests, the Murphy-Sturm rat lymphosarcoma was used. Forty-eight hours after the tumor was transplanted to forty-five rats, they were divided into three groups. Group A served as control and was not treated. Group B was given Mitomycin C, 1.5 mg./kg. wt./rat/day every second day, seven injections. Group C received the same dose of Mitomycin C with hematoporphyrin, 2.5 mg./100 gm. wt./rat/day administered two hours prior to Mitomycin. The body weight was taken before and two days after the treatment was completed. The tumors were re-

moved two days after the last Mitomycin C injection. Table IV summarizes the data so obtained.

TABLE IV

EFFECT OF HEMATOPORPHYRIN ON MURPHY-STURM LYMPHOSARCOMA

Group A. 15 rats, males, control, Murphy-Sturm lymphosarcoma, 48 hour transplant.

Group B. 15 rats, males, Murphy-Sturm lymphosarcoma, 48 hour transplant. Mitomycin C, 1.5 mg./kg. wt./day/rat every second day, seven injections.

Group C. 15 rats, males, Murphy-Sturm lymphosarcoma, 48 hour transplant. The same dose of Mitomycin C. Hematoporphyrin, 2.5 mg./100 gm. wt. injected two hours prior to Mitomycin injections.

Groups	Initial Wt. av. gm.	Final wt. 15th day av. gm.*	Tumor wt. 15th day av. gm.*	Mortality Rate %
Group A				
15 rats control tumor	110.4	148.5	12.345	13.3
Group B				
15 rats Mitomycin C total dose 10.5 mg./kg. wt./ rat in 14 days	108.6	82.3	1.25	60
Group C				
15 rats Mitomycin C total dose 10.5 mg./kg. wt./ rat in 14 days and HP, total dose 17.5 mg./ rat, 14 days	109.5	95.3	0.87	40

* Only the survived rats are included in the tabulation.

The analysis of the figures of Table IV suggests that the activation of the R.E.S. by intravenous injections of hematoporphyrin did not interfere with the oncostatic activity of Mitomycin C, and, in fact, it was somewhat enhanced. Although the hematoporphyrin application prevented leucopenia induced by Mitomycin C, it had no significant effect on the loss in weight, and on the general condition of the animals. They were emaciated in both groups, B and C. Thus the activation of the R.E.S. by hematoporphyrin does not entirely solve the problem of Mitomycin C cytotoxicity.

Comment: The activation of the R.E.S. by administering hemato-

porphyrin two hours prior to Mitomycin C injections, resulted in prevention of leucopenia and neutropenia in rats, with tumor tissue moderately sensitized by this substance. There was, however, a loss in weight both in the hematoporphyrin-Mitomycin C and Mitomycin C groups.

2. *Congo Red.* Adler and Reimann (1) were the first to introduce Congo red for testing the state and activity of the R.E.S. Congo red, an electronegative colloidal dye, has affinity for the histiocytes, and is deposited mostly in these cells shortly after its intravenous injection. Wedekind *et al.* (51) reported that Congo red has some hemostatic properties, shortening the coagulation time and increasing the platelet number. Waldorp and Alvarez (50), Massa and Zolezzi (20), Behr (6) and others confirmed the activating effect of Congo red on the R.E.S. Arons and Sokoloff (2) showed that the amount of Congo red injected was an essential factor as far as the effect of the dye on the R.E.S. was concerned. Large doses of Congo red, exceeding 10 mg./100 gm. wt., might blockade the system instead of activating it. In their investigation, cancerous rats and mice given Congo red revealed that a 3 mg./100 mg. wt. Congo red dose had a moderate inhibiting effect on transplanted tumors.

In the present study, the effect of Congo red on the toxicity of Mitomycin C was investigated, following the same pattern of testing as in previous experiments. For the toxicity tests, Mitomycin C, 1.5 mg./kg. wt./rat/day was given for six consecutive days, and Congo red, 2 mg./100 mg. wt./rat/day, three injections, prior to Mitomycin C administration. In the test on the effect of Congo red-Mitomycin C combination, a seven-day consecutive application of Mitomycin C was used, with four intravenous injections of Congo red. Tables V and VI summarize the data so obtained.

The analysis of the data presented in Tables V and VI indicate that Congo red has a much lesser effect on the Mitomycin C induced leucopenia than hematoporphyrin. Leucopenia seemed to be less pronounced when Congo red was injected prior to Mitomycin C but the difference between the Groups B and C was not striking. The loss in weight was somewhat smaller in the Congo red-Mitomycin C group, but then again, there was no indication that Congo red sensitized the tumor tissue and that oncostatic activity of Mitomycin C was enhanced by Congo red injections.

TABLE V

EFFECT OF CONGO RED ON THE TOXICITY OF MITOMYCIN C

Group A. 10 rats, males, Mitomycin C, 1.5 mg./kg. wt./rat/day for six consecutive days.

Group B. 15 rats, males, Mitomycin C, 1.5 mg./kg. wt./rat/day for six consecutive days and three intravenous injections Congo red, 2 mg./100 gm. wt./rat/day, two hours prior to Mitomycin C administration.

Groups	Initial Wt. av. gm.	Final wt. 8th day av. gm.*	WBC initial av.	WBC final 8th day av.*	Mortality Rate %
Group A					
10 rats Mitomycin C total dose 9 mg./kg. wt./ rat in 6 days	102.4	83.4	6,540	3,120	30
Group B					
15 rats Mitomycin C total dose 9 mg./kg. wt./ rat in 6 days and Congo red total dose 8 mg./100 gm. wt./ rat in 6 days	105.6	92.3	7,120	4,850	13.3

* Only the survived rats are included in the tabulation.

Comment: Congo red, injected intravenously prior to Mitomycin C, moderately decreased leucopenia and the loss in weight in treated rats.

SH-AMINO ACIDS AS PROTECTIVE FACTORS

Sokoloff *et al.* (44) reported that the glutathione liver content of rats receiving heavy doses of Mitomycin C was decreased, as compared with control. They also detected traces of fibrinolysin in treated animals. The work of Guest *et al.* (18) and others emphasized the inhibiting effect of cysteine on fibrinolysin. Accordingly, a series of tests with cysteine and cysteamine were conducted on rats given Mitomycin C. The amino acids were administered orally, 1.0 mg./kilo weight/rat/day for ten days, two days prior, six days during and two days after the Mitomycin C therapy course. In view of the fact that leucopenia induced by the antibiotic could be prevented either by bone marrow transplantation or by hematoporphyrin, our attention

TABLE VI

EFFECT OF CONGO RED-MITOMYCIN C ON MURPHY-STURM LYMPHOSARCOMA

Group A. 10 rats, males, control, with Murphy-Sturm lymphosarcoma.

Group B. 10 rats, males, with Murphy-Sturm lymphosarcoma, Mitomycin C, 1.5 mg./kg. wt./rat/day for seven consecutive days.

Group C. 15 rats, males, with Murphy-Sturm lymphosarcoma, Mitomycin C, 1.5 mg./kg. wt./rat/day for seven consecutive days and Congo red, 3.0 mg./100 gm./rat/day, four injections, two hours prior to Mitomycin C injections.

A one-day-transplant

Groups	Initial Wt. av. gm.	Final wt. 8th day av. gm.*	Tumor wt. 8th day av. gm.*	Mortality Rate %
Group A				
10 rats control tumor	150.4	171.2	11.23	—
Group B				
10 rats Mitomycin C total dose 10.5 mg./kg. wt./rat in 7 days	151.3	128.3	1.25	30
Group C				
15 rats Mitomycin C total dose 10.5 mg./kg. wt./rat in 7 days and Congo red total dose 8 mg./rat in 7 days	159.3	138.4	1.37	20

* Only the survived rats are included in the tabulation.

was directed chiefly to the loss in weight. The results of these tests are summarized in Table VII.

From the figures of Table VII, one may conclude that both cysteine and cysteamine gave considerable but not complete protection against toxic effects of Mitomycin C as measured by the loss in weight. The control, non-treated, rats gained 26 per cent of their weight during the ten-day period. The Mitomycin C treated group lost an average of 15 per cent; the Mitomycin C-cysteine group gained 10 per cent, and the Mitomycin C-cysteamine group increased its average weight by 12 per cent.

TABLE VII

EFFECT OF CYSTEINE AND CYSTEAMINE ON THE TOXICITY OF MITOMYCIN C

Group A. 10 rats, control, no treatment.
Group B. 10 rats, Mitomycin C, 1.5 mg./kg. wt./rat/day for six consecutive days.
Group C. 10 rats, Mitomycin C, 1.5 mg./kg. wt./rat/day for six consecutive days, and Cysteine, 1.0 mg./kg. wt./rat/day for ten days (2 prior, 6 during and 2 post-treatment) orally.
Group D. 10 rats, Mitomycin C, 1.5 mg./kg. wt./rat/day for six consecutive days, and Cysteamine, 1.0 mg./kg. wt./rat/day for ten days (2 prior, 6 during and 2 post-treatment) orally.

Groups	Initial Wt. av. gm.	Final wt. av. gm. after 10 days
Group A		
10 rats control No treatment	99.8	126.1
Group B		
10 rats Mitomycin C, total dose 9.0 mg./rat, six days	100.2	85.6
Group C		
10 rats Mitomycin C, total dose 9.0 mg./rat, six days and Cysteine, 10.0 mg./kg. wt. 10 days	99.2	109.1
Group D		
10 rats Mitomycin C, total dose 9.0 mg./rat, six days and Cysteamine, 10.0 mg./kg. wt. 10 days	98.3	108.1

Comment: Both cysteine and cysteamine gave some protection against toxic influence of Mitomycin C, as evidenced by gain in weight.

NECROSIN

In our investigation on the toxicity of Mitomycin C on rats and mice, it was observed that the toxic effects of this antibiotic were more pronounced in rats and mice, bearers of advanced tumors, than in normal animals or those with an incipient tumor. This observation was interpreted to mean that in the first instance, there are possibly products of injured and destroyed cells, absent, or present in a smaller quantity, in normal animals receiving Mitomycin C. The histologic

examination of the spleen of chicks, given large doses of Mitomycin C, showed not only shrinkage, as reported by us previously, but also necrotized areas in the central part of the organ. In our tests with intradermal injections of Mitomycin C to rabbit adbomen, followed by trypan blue intravenous injections, a similar necrosis was observed twenty-four hours after the Mitomycin C injection. In this case, the central part of the injected area was colorless, with dye accumulated on its periphery. The histologic examination revealed in this case a clearly defined necrosis of the epidermis. All these facts seemed to indicate that in Mitomycin therapy, particularly of advanced tumors, there might be present a necrotizing factor or factors, not only localized, but possibly in the circulatory system as well. The presence of such a factor, resulting from Mitomycin C application might explain the observations that necrotizing nephrosis was observed both in animals and humans treated with this antibiotic. Thus an attempt was made by us to detect the presence of such, or a similar factor in the spleen or the liver of animals given heavy doses of Mitomycin C.

Menkin (23) was the first to show that the euglobulin fraction, particularly of acid inflammatory exudates, contained a factor which he termed necrosin. Menkin (23, 24, 25) also demonstrated that this factor induced lymphatic blockade, caused by fibrinous plugs or thrombi within lymphatic vessels and that it shortened clotting time. Schwab and Watson (38) expressed the belief that necrosin might perhaps be identical to thromboplastin. Gorkin (15, 16, 17) in his extensive investigation of the nature of necrosin, was inclined to conclude that this factor is a pseudoglobulin of an enzymatic nature. In our exploratory investigation along these lines, we used the technique of Gorkin (16) as more suitable for our purposes than Menkin's method of bioassaying (23) which is based on the lymphatic blockade. We have described elsewhere in detail (45) the modified Gorkin method employed by us and which we found satisfactory for estimation of a necrosin-like substance in animal tissue.

In the first series of tests, we investigated the inflammatory exudate, induced by intradermal injection of Mitomycin C to rabbit abdomen. The factor, necrosin, was extracted following Gorkin's technique and it showed the same characteristics as those described by Gorkin. The substance was unstable, losing its activity after several days in storage, and it induced a typical necrosis in mouse skin, 24 hours after the

injection. Thus the inflammatory exudate induced by Mitomycin C contained a necrotizing factor similar to the one present in bacterial inflammatory exudate.

In brief, the procedure for estimation of necrosin-like substance content in tissues was as follows. Ten large rats, average weight 280 gm. with advanced tumor, Murphy-Sturm lymphosarcoma or Crocker carcinoma, were given Mitomycin C, a total dose 18 mg./kilo weight/rat in six days. The rats were killed, their livers and spleens removed, macerated, and the pH brought down to 6.6 with dilute lactic acid. The material was first purified with BuOH, centrifuged, and saturated with $(NH_4)_2SO_4$, 0.3. The precipitate was discarded, and the solution brought up to 0.5 saturation. Purified 18-fold, the active factor was obtained on electrophoresis, pH 8.2. The purity of necrosin was checked by the content of phosphorus, or rather its absence. There was, as a rule, traces of P, about 0.06%. The necrosin or necrosin-like substance, when injected intradermally to mice, gave a necrotized area of 6 mm. in diameter, 24 hours after the injection.

Our attempt to detect a necrotizing factor in the organs of normal animals given large doses of Mitomycin C were not successful. But in the case of rats with advanced tumors and which received heavy doses of Mitomycin C, we were able to isolate a factor which induced necrosis in mouse skin. This factor seemed to differ from Gorkin's necrosin, as being more thermostable, and resistant to heat at 56° C. We estimated that the spleen of rats, bearers of advanced tumors, and given 18 mg./kilo weight Mitomycin C contained an average 0.025 mg./1.0 gm. dry tissue, and the liver, 0.015 mg./1.0 gm. dry tissue.

Comment: The inflammatory exudate, caused by intradermal injections of Mitomycin C contains a necrotizing factor, similar to the one isolated from bacterial inflammatory exudates.

Discussion

There prevails among carcinologists an opinion, that a non-toxic oncolytic factor should be discovered, sooner or later. All the present research in the cancer field is actually directed to this goal. So far, however, no indication, no sign of a non-toxic drug which would inhibit spontaneous advanced cancerous growth, is in evidence. Unfortunate as it is, all antitumor drugs, used in chemotherapy of cancer are toxic to such a degree that no proper and rational treatment of cancer

patients is visible so far. One often wonders if the toxicity of an antitumor drug is not an inseparable part, an inbred property of its activity. For as Albert Fisher once emphasized, the chemical and enzymic differences between a normal and a diseased cell are very small indeed. This smallness of differences in the physiological deviations between a normal and diseased cell renders the obstacles to an effective chemotherapy of advanced cancer almost an insurmountable problem. Yet if we are unable so far to develop a non-toxic antitumor drug, we are still in a position to decrease the toxic side effects caused by known drugs, as we attempted to do with some degree of success in our present study on the cytotoxicity of Mitomycin C.

Summary

The cytotoxicity of Mitomycin C was investigated and an attempt was made to minimize it or prevent it by various physiological measures.

The inoculation of 100 or 125 million nucleated cells to rats receiving near-toxic doses of Mitomycin C prevented leucopenia and decreased the mortality rate of animals.

Splenectomy of rats treated with Mitomycin had no influence on the toxic side effects of this treatment.

The spleen extract containing the antihemorrhagic factor, TCRA, had a moderate beneficial effect on the toxic manifestations caused by Mitomycin C.

Mitomycin C, 0.02 mg., or a larger dose, injected intradermally to rabbit abdomen, induced an acute inflammatory process, with trypan blue being accumulated in the injected areas.

Blockade of the R.E.S. by intravenous injection of saccharated iron oxide (15 mg./kilo weight/rabbit) resulted in a delayed skin reaction of a hemorrhagic nature. However, no typical Schwartzman-like phenomenon was observed with Mitomycin C.

Blockade of the R.E.S. in rats given Mitomycin C by saccharated iron oxide (25 mg./kilo weight) resulted in accentuated leucopenia.

Activation of the R.E.S. in rats, given Mitomycin C, by hematoporphyrin (25 mg./kilo weight), resulted in prevention of leucopenia.

Rats receiving Mitomycin C and given orally cysteine and cysteamine (1.0 mg./kilo weight) showed a lesser loss in weight than the rats on Mitomycin C alone.

The inflammatory exudate, caused by intradermal injections of Mitomycin C, contained a necrotizing factor, similar to the one isolated from bacterial inflammatory exudates.

The problem of a necrotizing factor present in tumor-bearing rats, treated with Mitomycin, was discussed.

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MITOTIC ACTIVITY AND RATES OF GROWTH IN REGIONS OF ORAL EPITHELIUM DIFFERING IN WIDTH*

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Received February 23, 1960

During the last decade it has become widely accepted that the integrity of lining and covering epithelia is maintained by the continual replacement of cells rather than through the renewal of cellular constituents (Leblond and Walker, '56; King, et al., '59). Leblond has suggested that the proliferative capacity of the cells of the germinative layer is retained in the adult organism as an evolutionary adaptation to the continual injury to which covering and lining epithelia are subjected. The finding of continuous cell division in tissues with a constant total number of cells has raised the problem of how this process is controlled. While experimentally induced changes in the rate of cell turnover have been studied (for references see Bullough, '52; Pinkus, '51 and '54), little is known about many aspects of the physiologic process itself (Hoffman, '53; and Leblond and Walker, '56). The present study of the mitotic activity in different regions of the oral mucosa of mice was undertaken as a contribution to such knowledge.

1. The *distribution* of dividing cells has previously been studied in tumors and in regenerating liver (Hoffman, '53, p. 55 ff. and Brues and Marble, '37). It has been found that in these growing tissues the cells divide independently of one another and that the mitotic frequencies of tissue samples follow a curve of the Poisson type. In the present study, the frequency distribution of mitosis was analyzed in a tissue with a constant total number of cells.

2. A rather wide *range of mitotic rates* is found when corresponding epithelial regions are studied in several animals of the same strain,

* This investigation was supported in part by research grants C-3029, C-2705 and D-513 from the U.S. Public Health Service, Division of Research Grants, National Institute of Health, Bethesda, Maryland.

even if the conditions of testing are kept uniform (see, e.g., the coefficients of variation and standard errors in Henry, et al., '52 and Bullough, '49). The cause of variability may in part be the small size of the samples, since with increasing size of sample the range narrows. However, the possibility exists that the rate of cell turnover is a biological constant like body size or temperature, which varies from one individual to another. If this is the case, animals with a high mitotic rate in one region should have a high rate in a different region also. In order to test this, pairs of regions from the same animal were studied.

3. Whereas turnover times of epithelia have often been estimated from the determination of mitotic indices and a determination or a reasonable assumption concerning the duration of cell division, estimates of *rates of growth* have rarely been made (Leblond and Walker, '56). We do not know whether a thick epithelium grows faster than an otherwise similar thin one, since measurements of thickness have not been made in conjunction with mitotic studies. The rate of growth could, however, be estimated from the turnover time if the thickness were known. In the present study, estimates of the rates of growth of four epithelial regions of different thickness will be presented.

Material and Methods

Ten male mice of Strong A albino strain, 60 days old, were used. The animals were killed by decapitation at 2 P. M. The head was freed of skin and of most of the masticatory muscles. Part of the tongue was removed, and the oral cavity was propped open to permit the fixing solution to reach all areas rapidly. A one to two dilution of Bouin's solution was used for fixation. The time interval between decapitation and exposure to the fixative was no more than five minutes.

After fixation the heads were decalcified in sequestrine at pH 7.0, dehydrated and embedded in paraffin. Serial sections of 6 μ thickness were cut of five heads in the sagittal and five heads in the frontal plane. Every fifth section was stained with iron hematoxylin. The omission of four intervening sections was found to prevent the double counting of those dividing cells which were in more than one section. The staining procedure was so adapted that nuclei, especially of cells in mitosis, could be identified with ease and that the cellular layers of

the epithelium were clearly demarcated from the connective tissue underneath and the keratin above.

Quantitative studies were made on four regions. In the order of increasing thickness these were (1) a region in the floor of the mouth, (2) the central portion of the palatal mucosa (raphe), (3) a lateral portion of the palatal mucosa, and (4) a region in the cheek. These four regions included the thinnest and thickest epithelium of the oral cavity. The boundaries were so chosen that the cellular thickness within the included portions was as uniform as possible and that they could be identified in every histologic section.

Region 1 was a trapeze-shaped portion of the floor of the mouth, bounded by the openings of the sublingual ducts anteriorly, the lateral borders of the ducts at the sides and the attachment of the lingual frenulum posteriorly. It was studied in frontal sections.

Region 2, the central portion of the hard palate, was limited laterally by the elevations of the palatine molar rugae and included the area between the first and the last ruga. It was studied in sagittal sections.

Region 3, the lateral portion of the hard palate, covered the second, third and fourth of the molar rugae. Its lateral border was the gingiva. It was studied in saggital sections.

Region 4 was the portion of the cheek facing the molars. The region was studied in frontal sections. The included portion extended from the thick epithelium in the occlusal plane to the transitions between thick and thin epithelium near the upper and lower vestibules.

Mitotic rate. In the floor of the mouth and the cheek, the mitotic rate was determined directly. All nuclei in the epithelium between the boundaries were counted and the number and location of the mitoses recorded. In the lateral and central portion of the palate, the mitotic index was calculated from a count of the cells in mitosis and an estimate of the total number of cells in interphase. The estimate was made as follows: Drawings were made of projected sections (Mag. ×100). In each drawing, areas of a size to contain several hundred cells were mapped out according to the occurrence of land marks which made it possible to identify the precise boundaries under the microscope. The surface of these sample areas was measured in the drawings with the use of a planimeter. A count of the nuclei in each sample area was made in the microscopic section. The counts of the

cells per unit sample area were averaged and so the representative cell density in the central and lateral portions of the palate determined. The total cell number was calculated from the cell density per unit area and from the total area. The latter was estimated by planimetry on the drawings of the projected sections. In the central portion of the palate, every section was drawn and measured. In the lateral portion of the palate, it was found sufficient to draw and measure every fifth of the mounted sections, because measurements in adjacent sections proved to be nearly identical. Comparison of the estimates of mitotic rates with direct determinations showed the combined errors of the approximations to be within about 10%.

Distribution of Mitoses. In the lateral portion of the palate, 145 histologic sections were available, containing per section between 2928 and 4914 cells and from one to 15 mitoses. For purposes of statistical analysis, the numbers of mitoses per section were converted to numbers per 3,000 cells. In the floor of the mouth, center of palate and cheek, the distribution of mitoses could not be studied in the individual histologic sections, since these sometimes consisted of only 2–3 hundred cells and often contained no mitoses. In such instances, two to four of the adjacent mounted sections were combined into sampling units. The cell content of these tissue slices varied between 925 and a little over 2,000 cells.

Thickness of cellular layer. The thickness of the epithelium was measured on the histologic sections. In the sections through the floor of the mouth and through the central portion of the palate, micrometer readings were taken in the center and near the two ends. In the lateral portion of the palate, readings were taken at the crests of the rugae and in the intervening valleys. The readings per section were averaged to obtain the representative thickness of the section. The configuration of the buccal epithelium is too irregular for a limited number of measurements to yield a representative thickness. The sections of the cheek were therefore projected at a 1:100 magnification and the outlines of the cellular portions traced. On these tracings, the surface areas of the cellular portions were determined by planimetry and the lengths measured. The quotient of surface area and length was considered as the average thickness of the section.

FINDINGS

1. *Distribution of mitoses in the tissue samples (Table 1)*

Center of Palate. Nineteen samples of tissue, containing between 1077 and 1929 cells, were available. One contained no dividing cells, the other 18 had between one and 5, or mitotic indices ranging between .59 and 3.69. The frequency distribution of the indices was somewhat asymmetrical, but the class containing the average had the highest frequency. The indices of each of the five animals were scattered over the larger part of the range.

Floor of the Mouth. Twenty-six samples of tissue, containing between 925 and 2126 cells, were examined. The number of dividing cells varied from 1 to 7 per sample. The range of mitotic indices was 0.49 to 4.33. The frequency distribution of the indices was irregular and without a marked central tendency.

Cheek. Of the cheek, 58 samples of tissue, containing from 925 to 2075 cells, were examined. The number of dividing cells in these samples varied between 0 and 10, or 0 to 7.37 per 1,000 cells. This range was nearly twice as large as in the other areas. The frequency distribution was irregular, with some degree of clustering to both sides of the average. Each of the five animals made unevenly scattered contributions to indices in most parts of the range.

Lateral Palate. Of this region, 145 histologic sections, containing between 2,928 and 4,914 cells, were examined. The number of mitoses per section varied between 1 and 15, or from .25 to 4.36 per 1,000 cells. The frequency distribution of the mitotic indices was rather regular, and average, mode, and median index were close together. All five animals had similar individual distributions.

Fit of Poisson's Distribution. In the lateral portion of the palate, the number of dividing cells in a population of 3,000 ranged from 1 to 13 and averaged 5.35. Assuming random occurrence of cell division, the probability of being in interphase for any cell in a given section was $\frac{2994.65}{3000}$, the probability of being in mitosis was $\frac{5.35}{3000}$. The expected proportional frequencies of sections containing respectively 0, 1, 0, . . . 13 mitoses were obtained by expanding the term

$$F = \left(\frac{2994.65}{3000} + \frac{5.35}{3000}\right)^{3000}.$$

TABLE 1
DISTRIBUTION OF MITOSES IN THE TISSUE SAMPLES

Mitotic Index	0.0	.33	.67	1.0	1.33	1.67	2.0	2.33	2.67	3.0	3.33	3.67	4.0	4.33 and over
	NUMBER OF SAMPLES													
Center of Palate	1	0	3	2	2	5	1	1	1	0	1	1		
Floor of Mouth			4	2	2	4	4	2	2	2	3	1	0	1
Cheek	2	0	2	4	9	2	8	8	6	4	2	4	0	6
Lateral Palate		9	9	14	26	25	21	14	16	5	2	2	1	

The expected absolute frequencies in a total of 145 sections are shown in the hollow circles of Fig. 1. The observed frequencies, which are shown in the solid circles of Fig. 1, showed close agreement with the theoretical values. The expected variance was 5.35, the observed value, 5.04.

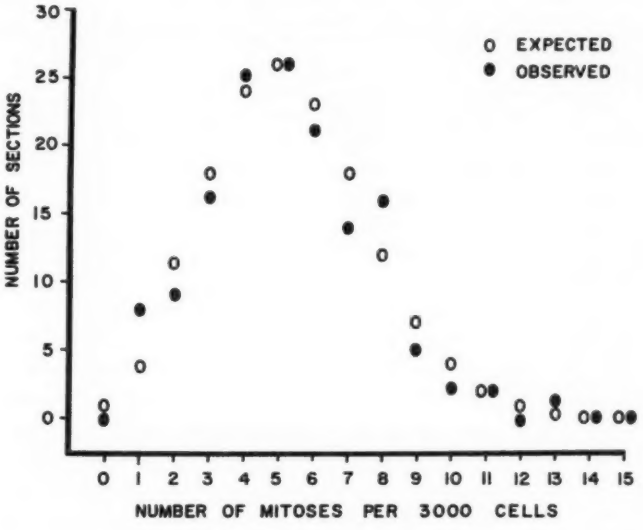

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FIGURE 1

Observed and expected frequencies of tissue sections of the lateral portion of the palate containing from 0 to 15 dividing cells. Expected frequencies computed on the assumption that the distribution of mitoses follows Poisson's law.

A larger set of samples would probably have shown that the distribution of mitoses in the epithelium of the center of the palate fitted a curve of the Poisson type equally well as in the lateral portion. The wide range of indices in the buccal epithelium, which was found in every animal, does not fit a single curve of the Poisson type, but might be the summation of two random distributions with different averages. The same may apply to the epithelium in the floor of the mouth.

2. *Comparison of pairs of mitotic rates in the same animal*

The mitotic rates in the cheek and in the floor of the mouth were determined in one set of 5 animals, and the rates in the lateral and in the central portion of the palate in a second set of five animals (Table 2).

TABLE 2
MITOTIC INDICES IN PAIRS OF REGIONS OF THE SAME ANIMAL

Mitotic Index			Mitotic Index		
No. of Animal	Buccal Epithelium	Floor of Mouth	No. of Animal	Lateral Region of Palate	Central Region of Palate
38	1.83	1.39	35	1.30	1.88
40	2.53	1.92	31	1.43	1.02
39	2.60	2.36	32	1.73	1.99
41	2.76	1.92	36	2.05	2.60
48	3.22	2.78	33	2.14	1.17
Averages and standard errors	2.59 ± 0.25	2.07 ± 0.26		1.73 ± 0.18	1.73 ± 0.32
Average number of cells per animal	16,850	7,760		90,800	5,850

In all four regions, the five animals showed considerable differences in their mitotic rates. These differences were in part related to the size of the tissue samples. The range in the lateral region of the palate, where more than 90,000 cells per animal were examined, may be taken as indicative of the degree of inter-individual variability in this region. Here the difference between the highest and lowest rate was about 65%. In the central region of the palate, only about 6,000 cells per animal had been available. The highest mitotic rate was about 2.5 times as high as the lowest one, and the relative standard error of the mean rate for the five animals was almost twice as large as in the lateral region. In both cheek and floor of the mouth, the highest mitotic rate was twice as high as the lowest one. The relative standard errors of the means were unrelated to the size of the samples, being only a little higher in the floor of the mouth than in the cheek.

In Table 2, the first set of five animals was arranged in the order of increasing mitotic rates in the buccal epithelium, the second column listing the rates of the floor of the mouth of the corresponding animals. It can be seen that the rates in the floor of the mouth go up in

nearly the same ascending order and to a similar degree as those in the buccal epithelium, indicating a high degree of correlation in these two distant epithelial regions.

Although the second set of five animals averaged the same mitotic rate in the two palatal regions, there was no correlation of the individual rates. This may be due to the small number of cells in the central region of the palate.

3. *Thickness of the cellular layer*

The individual micrometer readings of cellular thickness differed within a range of 100% or more in the sections of all four regions. The averages per histologic section were obtained from 2-6 readings at individual points. In these averages, the effect of the irregularly projecting epithelial ridges and of the intruding papillae of connective tissue was cancelled out to some extent. The regional boundaries had been carefully selected to ensure internal uniformity, and the difference between thinnest and thickest section was of the order of 50% in all four regions. This variability is slight in comparison to the tenfold range of variation in the mitotic indices of the same sections. The variation in thickness had no anatomical gradient, but was due to randomly distributed irregularities.

Averages Per Animal. In the epithelium of the floor of the mouth, between 7 and 13 sections per animal were measured; in the central portion of the palate, between 4 and 8; in the lateral portion 8 to 18, and in the buccal epithelium 10 to 11. The standard errors of the means of the groups were unrelated to the numbers of sections measured.

The regional averages ranged from 21.2 ± 0.75 μ in the floor of the mouth to 116.5 ± 9.89 μ in the buccal epithelium (Table 3). The individual animals were far less variable in the widths of the homologous epithelia than in their mitotic rates. Even in the buccal region, where the configuration of the epithelial ridges is extremely irregular and where one animal had an exceptionally thin epithelium, the difference between the highest and the lowest value was only 55%. In this region, the standard error was 8% of the group average, in the other three, it was between 1.7 and 2.7%.

In view of the small range of inter-individual variation, it was surprising that there was some degree of correlation in the widths of the

TABLE 3
THICKNESS OF CELLULAR LAYER (μ)

No. of Animal	Floor of the Mouth	Cheek	No. of Animal	Center of Palate	Lateral Palate
48	18.7	87.9	36	45.2	62.8
40	20.6	132.9	32	45.3	63.5
39	21.5	136.1	35	47.9	62.7
41	22.5	106.9	33	48.3	70.9
38	22.5	118.9	31	48.4	65.5
Averages and standard errors	21.2 ± 0.75	116.5 ± 9.89		47.0 ± 0.81	65.0 ± 1.73

two regions which came from the same animal. The animal which had the thinnest buccal epithelium had also the thinnest epithelium in the floor of the mouth. In two animals both palatal regions were of below average thickness; in two other animals, both were of above average thickness.

4. *Location of mitoses (Table 4)*

Of 81 mitoses in the epithelium of the *floor of the mouth,* 79 were in the basal layer and 2 in more superficial cells. In the *center of the palate,* 48 of 52 mitoses were in the basal layer, and 4 in the adjacent spinous layer. In the *lateral portion of the palate,* 581 mitoses were located in the basal layer, and 356 in more peripheral cells. Nearly two-thirds of the latter were in the deepest spinous layer and about one-third in the adjacent layer of spinous cells. No mitoses were seen peripherally to the fifth layer of spinous cells. In the *cheek,* 188 of 207 mitoses were in the basal layer, 15 in the adjacent and 4 in the next row of cells.

TABLE 4
LOCATION OF MITOSES IN DIFFERENT EPITHELIAL REGIONS

Region	% of Mitoses Above Basal Layer	Mitotic Index	Thickness of Cellular Layer (μ)
Floor of Mouth	2	2.07	21.2
Center of Palate	10	1.73	47.0
Lateral Portion of Palate	37	1.73	65.0
Buccal Epithelium	9	2.59	116.5

The lateral portion of the palate, in which a third of the mitoses occurred above the basal layer, had the largest number of rows of spinous cells, the cells in this region being smaller and flatter than in the buccal epithelium. Thus the number of stages of differentiation seemed to be the determining factor in whether cells in the spinous stage still undergo division.

In the buccal epithelium, mitosis was substantially limited to the basal cells, just as in the center of the palate, despite the fact that the proportion of dividing cells was higher and that the width of the epithelium was more than twice as great. This of course means that the frequency of division is much higher among the basal cells of the cheek epithelium than among those in the center of the palate.

5. *Turnover times and rates of regrowth in the four regions*

Turnover Times. The ratio of the number of cells in mitosis and the number in interphase equals the ratio of the duration of mitosis and the average duration of interphase. The latter is identical with the turnover time of the cellular portion. On the assumption that cell division took one hour, the average turnover times were as follows:

TABLE 5
TURNOVER TIMES OF FOUR REGIONS OF ORAL EPITHELIUM

Region	Mitotic Index	Average Duration of Interphase = Turnover Time				
Floor of Mouth	$\frac{2.07}{1000}$	$\frac{X_1}{1}$	=	$\frac{1000}{2.07}$	hours;	$X_1 = 20.1$ Days
Center of Palate	$\frac{1.73}{1000}$	$\frac{X_2}{1}$	=	$\frac{1000}{1.73}$	hours;	$X_2 = 24.1$ Days
Lateral Portion of Palate	$\frac{1.73}{1000}$	$\frac{X_3}{1}$	=	$\frac{1000}{1.73}$	hours;	$X_3 = 24.1$ Days
Cheek	$\frac{2.59}{1000}$	$\frac{X_4}{1}$	=	$\frac{1000}{2.59}$	hours;	$X_4 = 16.1$ Days

Rates of Growth. The amount of growth required for the complete renewal of the cellular portion is of course determined by its width. By definition, the amount of growth during the turnover period equals the width of the cellular portion, and the amount per day equals this width divided into the turnover period. So estimated, the following daily rates of growth were obtained.

TABLE 6
DAILY RATES OF GROWTH OF FOUR REGIONS OF ORAL EPITHELIUM

Region	Turnover Time (Days)	Thickness of Cellular Layer (μ)	Daily Rate of Growth (μ)
Floor of Mouth	20.1	21.2	1.05
Center of Palate	24.1	47.0	1.79
Lateral Portion of Palate	24.1	65.0	2.70
Cheek	16.1	116.5	7.24

DISCUSSION

1. *Mitotic frequencies and control of mitotic rate*

In four regions of oral epithelium, the mitotic indices of tissue samples containing between about 1,000 and 4,000 cells varied over a seven- to ten-fold range. The scatter of indices was widest in the cheek and in the floor of the mouth and somewhat narrower in the two palatal regions.

The distribution of mitoses in histologic sections of the lateral palate was found to be in close agreement with a curve of the Poisson type, which is to say that there was chance incidence of the number of dividing cells in the tissue sections. This may be taken to indicate random timing of division and interphase.

The study of spermatogenesis has shown that other organs of rodents are capable of the exact opposite of randomness, that is, of precisely synchronized cell division (Roosen-Runge, '50 and Leblond and Clermont, '52 a and b). The cells of long segments of seminiferous tubules have been shown to be "in phase," advancing together from one stage of the spermatogenic sequence to the next and undergoing their divisions at the same time.

It is, on the other hand, not surprising to find random timing of cell division in unphysiologic situations in which the cells divide as rapidly as they can. A random distribution of dividing cells has been shown following partial hepatectomy (Brues and Marble, '37), when the liver has to make up a sudden loss of cells, and also in the growth of malignant tumors (Hoffman, '53, p. 55 ff.), which are not subject to the growth controlling mechanisms of their bearers. Cell division in stratified epithelia with exfoliative growth does not normally proceed

at the fastest possible rate. This is evident from the fact that even minimal injury at the surface of the cornified layer causes a sharp increase in proliferation at the base (Pinkus, '51).

The regulation of cell division in a synchronized organ can be conceived in a fairly simple way. Thus Leblond suggests that the ejection of sperm provides the stimulus which initiates a new cycle of spermatogenesis, mitoses occurring in the proper phases (Leblond and Walker, '56). Surface shedding and proliferation at the base must of course be in balance in the case of covering epithelia also. But, given the random timing of mitosis, the regulatory mechanism must consist in fixing the average intermitotic time of the participating cells.

The present study has shown that the daily rates of growth vary over a wide range in different epithelial regions, furthermore, that the basal cells carry the bulk of the cell divisions in each case. Therefore, the average interphase interval of the basal cells must vary greatly from region to region. It is possible that these variations are mediated by local differences in the vascular pattern, since a number of studies have suggested that the circulatory supply is a rate-limiting factor in epithelial cell division (see Bullough, '52 for references to his work; Pinkus, '51, and Marwah, et al., '60). A regulation of the over-all rate by systemic, e.g., hormonal influences may of course be superimposed on the basic local pattern, and a change in a local rate may be induced by a local injury or disturbance.

2. *Range of individual mitotic rates and of cellular widths*

The mitotic rates of the five animals were found to vary over a 1.65 fold range in the lateral portion of the palate, and over a two-fold range in the cheek and the floor of the mouth. The range in the center of the palate was wider, but was influenced by the small number of cells studied. The widths of the cellular portions of corresponding regions were much less variable, the range amounting to no more than a 10% variation in any region except the buccal epithelium.

The mitotic rates of both cheek and floor of the mouth, which had been determined in the same animal, varied in the same direction from one animal to the next. The same tended to be true for the width of the cellular portions of these two regions. In the two palatal regions, which had been studied in the same animal, the cellular widths were closely correlated. Due to the small number of cells in the center of

the palate, there was no correlation of the mitotic rates in center and lateral region.

Previous work had shown a similar surprising constancy of the width of the cellular portion at the ventral surface of the tongue in rabbits as was here observed in three regions of the oral cavity of mice (unpublished observations). The width of homologous regions of stratified epithelia is, of course, subject to a vast range of *species* variation. Our work suggests that the *individual* variation of this trait is slight for most regions. But if the present findings may be generalized, individual deviations from the species or strain average, however slight, are not confined to a single epithelial region, but characterize the stratified epithelia of the animal as a whole.

The same appears to be true of the rates of mitotic activity, again provided that the present findings are more generally valid. In the case of mitotic activity, the range of individual variation is much wider. The present study confirms many previous ones in this respect (cf. literature quoted in the introduction). By contrast, not only the thickness of the cellular portion, but also the duration of mitosis appear to be remarkably constant (Ris, '55). This being the case, the mitotic index of a given region is in direct proportion to the rate of regenerative growth of the region. Again, it appears that deviations from the species average may characterize the stratified epithelia of the individual as a whole. The wide range of individual variation in this respect might be one of the factors underlying different susceptibility to cancer.

3. *Relation between growth rate and thickness of epithelium*

While the turnover periods of the four epithelial regions varied over a narrow range, the daily rates of growth varied from about 1 μ in the epithelium covering the floor of the mouth to about 7 μ in the buccal epithelium.

Fig. 2 shows that there was an approximately rectilinear relation between the growth rates of the four regions and the widths of the cellular portions, growth increasing slightly faster than width. The four regions had been selected so as to include the extremes in width and also to represent the different types of keratinization encountered in the oral cavity of mice. The buccal epithelium forms a soft, flaky kind of keratin, that of the two palatal regions a harder, compact

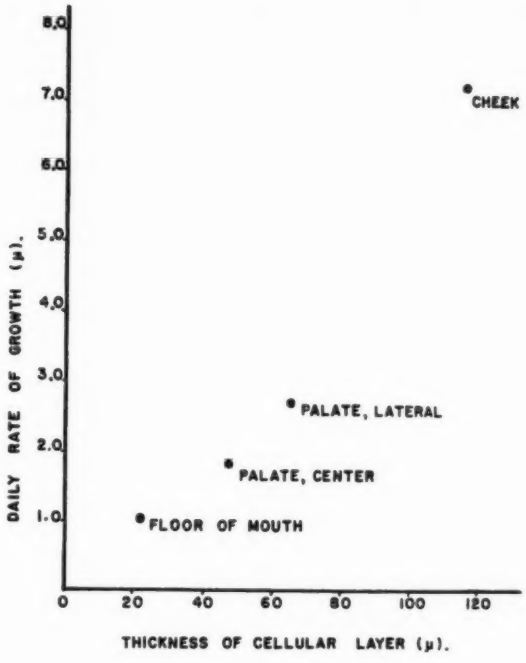

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FIGURE 2
Relation between width of the cellular layer and daily rate of growth in four epithelial regions.

kind. The keratin in the floor of the mouth is of a type intermediary in consistency and coherence (Weinmann, et al., In Press). The two regions with softer keratin have slightly faster growth rates than the two with harder keratin (Fig. 2). In all four regions, however, the growth rates are related essentially to the width and not to the type of keratinization. Therefore, it is likely that in the entire epithelium of the oral cavity of the mouse the rates of growth vary in approximate proportion to the width.

Why cellular width and growth rate are related in this way is a question which has not before been raised. Comparison of the four regions

showed that the thickness of both the stratum corneum and of the cellular portions varied in the same *direction*. It may well be that the growth rates vary in a *quantitative* relation both to the width of the stratum corneum as well as to the width of the cellular layer, but a study of this relation has not been completed yet. A correlation of the growth rate and the width of the stratum corneum would be less surprising than the observed relation of growth rate and cellular width; both rapid surface wear and a thick cornified layer might be expected at sites under mechanical stress. If one accepts this, then the question would be why the cellular portion also, and not just the stratum corneum alone, has greater thickness in such locations.

Summary

Mitotic indices, location of dividing cells, and thickness of the cellular layer of epithelial regions were determined in the oral cavity of 60 day old male Strong A albino mice. Four regions: the cheek in the occlusal plane, the floor of the mouth, a lateral and a central portion of the hard palate, mapped out for internal uniformity and reproducibility of boundaries, were examined. These areas included the extremes in width and all types of keratinization which occur in the oral cavity of mice. Turnover times and rates of growth were estimated from the mitotic indices and the thickness of the cellular layer.

The mitotic indices of tissue sections of the four regions varied over a seven to ten-fold range. In the largest region, the lateral part of the palate, a chance distribution of the indices in accordance with Poisson's curve could be demonstrated. In all four regions, mitoses occurred predominantly in the basal layer. Groups of five animals differed in a 1.65 to two-fold range in the average mitotic indices of homologous regions. The mitotic indices of two regions determined in the same animal deviated both in the same direction from the average of five animals.

The average width of the cellular portion ranged from about 21 μ in the floor of the mouth to about 117μ in the cheek. Homologous regions of individual animals differed but little from the average of five, while the widths of two regions determined in the same animal tended to deviate in the same direction from the average.

The turnover times of the cellular portions varied from 16 days for

the buccal epithelium to 24 days for the epithelium of the palate. The daily rates of growth, however, varied from about 1 μ in the floor of the mouth to more than 7 μ in the buccal epithelium.

It was concluded that:

(1) Control of the rate of cell division involves fixing the average intermitotic time of the participating cells and that this time span varies widely in different epithelial regions. It is suggested that differences in the local vascular pattern may mediate these local differences in the rate of cell division;

(2) A mitotic rate and cellular width higher or lower than the species or strain average may be traits characterizing all stratified epithelia of an individual.

(3) The growth rates of different epithelial regions vary in approximate proportion to the width of the cellular layer and perhaps also in a quantitative relation to the width of the keratin layer.

Acknowledgments

The help of Dr. Harry Sicher, Professor of Anatomy, Loyola Dental School, in the critical reading of the manuscript and the kind encouragement given to this work by Dr. Isaac Schour, Dean, College of Dentistry, University of Illinois, are gratefully acknowledged.

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Growth, 1960, **24**, 47-58

THE EFFECT OF SELECTION AND PLANE OF NUTRITION ON RELATIVE SIZE IN THE RAT[1]

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Received March 14, 1960

Natural and domesticated populations frequently occur in a wide variety of environments, some quite rigorous. In the presence of selection, either artificial or natural, the phenomenon of genetic-environmental interaction may be significant. It is important to know whether an environment favorable to the expression of a trait allows most rapid selection response, or if selection in another environment results in more rapid progress. Another question is whether or not the expression of a trait in one environment has a different genetic basis than in another.

Although the possible existence of genetic-environmental interactions has been clearly recognized and widely discussed for at least fifty years, genetic theory has not been fully developed and experimental data are not abundant. Hammond (1947) expressed the view that an environment most favorable to the expression of a desired trait would allow most rapid progress under selection; and if the improved population is then transferred to less favorable conditions, it will have attained a higher level of performance than could have been achieved by the same amount of selection under less favorable conditions. Falconer (1952) pointed out that the existence of genetic-environmental interactions invalidated these views. Falconer (1952)

[1] This investigation was supported in part by Cancer Research Funds of the University of California and was conducted in cooperation with the Animal Husbandry Research Division, Agricultural Research Service, U. S. Department of Agriculture, under Western Regional Project W-1, "The Improvement of Beef Cattle Through the Application of Breeding Methods."

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determined that, if only two environments are considered, the interaction between genotype and environment may be expressed as a genetic correlation. When thus formulated, computational procedures already available for dealing with genetic correlations may be applied. Falconer and Latyszewski (1952) found evidence of interaction between genotype and environment when mice were selected for 6-week weight under high and low planes of nutrition. Morley (1955) divided a number of half-sib groups of Merino sheep into two lots, one being exposed to a high plane of nutrition and the other to a low plane from 3 to 17 months of age. Sire-treatment interactions were considered a measure of genotype-environment interaction. Such interactions were large and highly significant for weight at 12 and 17 months, but not for Greasy fleece weight, yield, clean fleece weight, staple length, crimps per inch or for 6-month body weight. Lowry et al. (1956) investigated genetic merit for egg production traits of pullets housed in floor pens as compared with full sibs housed in individual cages. No evidence of genotype-management interaction was found.

Sokal and Hunter (1958) reported evidence of genetic-environmental interaction in Drosophila selected for long and short larval period, peripheral and central pupation site, and resistance and susceptibility to DDT in the larval mediums.

In view of the important implications for breeding practice as well as evolution, the need for further experimental evidence seems clear. An experiment was designed on the basis of Falconer's (1952) model to study the effect of selection and plane of nutrition on 70-day body weight and relative size in the laboratory rat. This paper presents an analysis of one phase of this experiment.

Source of Data

A base population was obtained by forming the four-way cross from four widely divergent, but highly inbred lines. Twenty pairs were randomly assigned each of two nutritional regimes ("Standard" and "Roughage"), and within each nutritional regime ten pairs were randomly designated "Select" and the other ten "Random." The four lines thus formed are referred to as Standard Select, Standard Random, Roughage Select and Roughage Random. The Standard nutritional regime is a complete ration developed in this laboratory which promotes normal growth. The Roughage ration is a mixture of 65

per cent standard and 35 per cent ground alfalfa, and was fed from weaning at 28 days through 70 days. The roughage ration resulted in a reduced 70-day weight, but the effects were dissipated by 100 days and reproduction was normal. Mass selection for 70-day weight was practiced in the select lines and a table of random numbers used to select individuals in the random lines. Random mating was practiced in all lines, except that full sib matings were prohibited. Each generation of each line consisted of ten pairs. Several extra pairs were mated in each line and used in the event of failure, which was rare. Each litter was reduced to six at five days, an attempt being made to retain 3 males and 3 females, but without selection within sexes.

Prior to the sixth generation each pair produced only one litter per generation. Females were mated when the youngest in each generation reached 100 days. Each generation was littered during an interval of one to four weeks, with representatives of all four lines approximately equally distributed over the interval.

The parents of the sixth generation were remated to produce a second litter which was treated in the same manner as the first. The data reported herein were obtained from these animals. There were 220 individuals from 38 litters distributed among two sexes and four lines, as indicated in Table 1.

The individuals of each litter were weighed and killed at seventy days. The data include: body weight, body length, body width, weight of the entire unwashed gastrointestinal tract, washed stomach, washed caecum and colon, washed small intestine, liver, heart and thyroids.

The rats were chloroformed until no heart beat could be detected, and were then stretched out dorsally. Body length was determined with a caliper as the distance from the anterior tip of the nose to the posterior edge of the anal aperture. Width was determined as the distance across the shoulders. All dissections were made by one of us (H.J.W.) and a uniform procedure with respect to time of killing, washing, blotting and weighing was followed throughout the experiment.

Analytical Method

Previous experience (Kidwell et al. 1959) indicated large differences between sexes in body weight. The limited numbers and unequal distribution among subclasses indicated a separate analysis for males and females rather than a single model including a sex term.

TABLE 1
MEANS OF MEASUREMENTS CLASSIFIED BY LINE AND SEX

Item	Standard-Select		Standard-Random		Roughage-Select		Roughage-Random	
	Males	Females	Males	Females	Males	Females	Males	Females
Number of litters	10		11		9		8	
Number of rats	25	30	32	33	28	26	24	22
70-day weight, gm.	296.6	207.4	249.1	158.8	246.3	183.1	210.8	166.2
Body length, cm.	21.7	19.5	20.9	18.3	20.9	19.0	20.1	18.5
Body width, cm.	5.36	4.86	5.46	4.47	4.98	4.60	4.67	4.38
Unwashed G.I. tract, gm.	27.52	19.79	21.93	14.62	27.94	19.87	26.60	20.65
Stomach gm.	1.59	1.30	1.33	1.07	1.78	1.47	1.48	1.28
Caecum and colon, gm.	3.75	2.91	3.30	2.35	4.22	3.27	3.73	3.05
Small intestine, gm.	9.74	7.77	8.88	6.04	9.26	7.19	8.81	7.22
Liver, gm.	15.40	10.50	13.08	9.30	12.83	9.40	10.60	9.04
Heart × 10, gm.	10.53	7.56	8.51	5.81	8.06	6.79	7.45	6.47
Thyroid, mg.	30.21	25.44	35.09	24.79	25.61	21.50	27.21	28.45

Bartlett's test of homogeneity of variance as described by Snedecor (1956) was applied the various traits. On the basis of these tests all data were transformed to logarithms to insure equal variances within subclasses. Morley (1956) and Ashoub et al. (1958) found logarithmic transformations necessary in similar studies with sheep and mice, respectively.

Since absolute organ size is a function of body size, each of the other traits was divided by body weight to determine its relative size. This was accomplished by subtracting the logarithm of body weight from the logarithm of each trait. In order to avoid negative logarithms the stomach weight was multiplied by a factor of 10 and heart weight by a factor of 100.

A 2×2 factorial experiment with disproportionate subclass numbers was employed to account for the two selection procedures, two nutritional regimes and the interaction between them. The interaction may be considered as being between genotype and environment to the extent that selection has produced genetic differences among the lines.

Results and Discussion

Phenotypic Variability

Means of the various traits classified by line and sex are presented in Table 1. The variances within subclasses and the result of Bartlett's test of homogeneity of variance (Snedecor, 1956) are presented in Table 2.

The unequal variances per se are of interest. Waddington (1942) is credited with the generalization that any radical departure from the genetical situation to which a species is adapted impairs morphogenetic regulation and hence causes an increase in phenotypic variability. Michie (1955) and McLaren and Michie (1956) formulated the corollary hypothesis that abnormal phenotypic variability can be elicited by subjecting developing organisms to unaccustomed environmental conditions, even when these conditions are uniform. Ashoub et al. (1958) cited a number of published analyses in support of this hypothesis of "environmental destabilization" and tested it by subjecting mice to hot (28° C), temperate (21° C) and cold (5° C) temperatures from birth through four weeks. Individual weights were recorded at weeks 1, 2, 3 and 4 and analyzed for the sexes separately. The mice raised at the extreme temperatures were much more vari-

TABLE 2
WITHIN TREATMENT VARIANCES

	70-day weight		Body length		Body width		Unwashed G.I. tract		Stomach	
	♂♂	♀♀†	♂♂*	♀♀*	♂♂	♀♀	♂♂†	♀♀†	♂♂	♀♀†
Standard Random	1,056.0	307.5	0.48	0.36	0.25	0.11	24.1	8.8	.03	.01
Standard Select	1,132.7	965.8	0.86	0.90	0.14	0.11	14.1	19.1	.03	.05
Roughage Random	1,441.1	459.0	1.02	0.69	0.20	.13	56.5	12.8	.04	.02
Roughage Select	611.8	900.7	0.34	0.48	0.12	.07	35.3	27.3	.05	.02

* Unequal variances at 5% level of probability.
† Unequal variances at 1% level of probability.

TABLE 2 (*Continued*)

	Caecum and colon		Small Intestine		Liver		Heart		Thyroid	
	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂†	♀♀†	♂♂†	♀♀*
Standard Random	.18	.14	1.4	0.9	4.6	4.3	1.3	0.2	91.5	45.5
Standard Select	.28	.19	1.4	1.4	5.2	4.1	3.1	1.6	39.3	23.1
Roughage Random	.37	.17	2.5	0.8	4.4	3.0	0.7	0.7	37.9	37.3
Roughage Select	.27	.23	1.7	1.7	3.6	4.2	2.0	1.5	22.6	19.5

able than those raised in the temperate environment. This was partly attributed to an exacerbation of the tendency for body weight to vary inversely with litter size; but was due in part to an increase of variability among litter mates. This "destabilizing" effect was more pronounced in the hot than in the cold environment.

The unequal variances in the present data are due to nutritional environment, selection procedure and the interaction between them. It is not possible to identify exactly the relative magnitude of each cause, and hence to determine whether they are due to genetic or environmental destabilization or both. It would appear, a priori, that the nutritional environment was more drastic in effect than the applied selection pressure. Inspection of the variances in Table 2 provides an indication of some trends.

Selection procedure and nutritional environment were designed to influence 70-day body weight. The effect of the ration was mediated through an increase of fiber and a decrease of energy concentration, and would, therefore, be expected to exert a major influence on the digestive tract and closely associated organs. It is of some interest to note that, with respect to body weight and stomach weight, only among the females were unequal variances found. An effect on the variances of the caecum and colon and the small intestine might have been expected and, conversely, there appears no a priori reason for expecting the variances of heart and thyroid weights to differ. Likewise, there is no apparent explanation for the effect on body length, but not width.

The unequal variances among the females, but not the males, for body and stomach weight suggests a sex difference, but there is no apparent explanation.

The hypothesis of genetic and environmental destabilization would suggest the smallest variance in the Standard-Random group and the largest variances in the Roughage-Select group, with the Standard-Select and Roughage-Random being intermediate and their relative size determined by the relative influence of selection and nutrition. Except for thyroid weight, among those traits with unequal variances, the variance of the Standard Random group is lowest or next to lowest. The Roughage Select group, however, generally does not exhibit the largest variance. There is, in fact, no generally consistent pattern in this respect.

These data provide evidence of genetic and/or environmental destabilization for some, but not all, of the traits studied. The data are insufficient to indicate a logical, consistent pattern in this respect.

Relative Size

Means of the logarithms of the traits and of the ratio of each trait to body weight, classified by ration, selection procedure and sex are presented in Table 3. The analysis of variance of the logarithms of the observations and ratios are shown in Table 4. In the presence of interaction, main effects have little or no meaning, hence mean squares are omitted.

Ration and/or selection procedure or interaction between them affected the actual size of all organs or size measurements. This is as expected since they are all highly correlated with body size. However, there was no evidence of treatment effect on the relative size of liver or heart.

The difference between the sexes is most striking. There are eleven instances of interaction among the thirty-eight analyses. Ten of these are among the females. The hypothesis of equal occurrence of interactions between the sexes is rejected at the .02 level of probability. The greater occurrence of interactions among the females could be the result of sex-linked genes or of physiological differences due to sex per se.

Selection for 70-day weight resulted in relatively shorter and narrower individuals in both nutritional regimes. Among the males it resulted in a decrease in the relative size of the thyroid.

The roughage ration increased the relative size of the components of the digestive tract, but except for two interactions in the females, selection was ineffective.

Summary and Conclusions

A 2×2 factorial experiment was used to estimate the effect of two selection procedures, two nutritional regimes and the interaction between them on relative organ size in the laboratory rat. A total of 220 individuals from 38 litters distributed among the two sexes provided the data. Observations included 70-day weight, body length and width, weight of unwashed gastrointestinal tract, washed stomach, caecum and colon, small intestine, liver, heart and thyroid and the ratio of each to body weight.

TABLE 3

MEANS OF LOGARITHMS OF MEASUREMENTS AND RATIOS CLASSIFIED BY LINE AND SEX

Item	Standard	Select	Standard	Random	Roughage	Select	Roughage	Random
	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀
Number of litters	10		11		9		8	
Number of rats	25	30	32	33	28	26	24	22
log 28-day wt.	1.87671	1.89778	1.85708	1.82658	1.90645	1.89008	1.84717	1.81488
70-day wt.	2.46703	2.31046	2.39341	2.19826	2.38883	2.25702	2.31744	2.21708
body length	1.33544	1.28779	1.31985	1.26194	1.31969	1.27703	1.30349	1.26734
body width	0.72793	0.68564	0.70653	0.64867	0.69586	0.66196	0.66710	0.63651
unwashed G.I. tract	1.43596	1.28370	1.33646	1.15656	1.43602	1.28393	1.40886	1.30828
stomach	1.19938	1.07472	1.12106	1.02612	1.24801	1.16618	1.16643	1.10571
caecum and colon	0.56797	0.45758	0.51503	0.36715	0.62216	0.51055	0.56618	0.47976
small intestine	0.98559	0.88526	0.91616	0.77459	0.96220	0.84958	0.93833	0.85864
liver	1.18221	1.01128	1.11081	0.95938	1.10321	0.96280	1.01689	0.94858
heart	1.97709	1.87097	1.93469	1.76228	1.90385	1.82538	1.86442	1.80723
thyroid	1.47117	1.39704	1.52854	1.37838	1.40089	1.32369	1.42404	1.44450
(body length/wt.)	0.86841	0.98003	0.92644	1.05170	0.93086	1.02001	0.98605	1.05026
(body width/wt.)	0.26010	0.37718	0.37563	0.52212	0.30703	0.40494	0.37001	0.41944
(G.I. tract/wt.)	0.92653	0.94294	0.94305	0.95829	1.04719	1.02684	1.09143	1.09120
(stomach/wt.)	0.73235	0.80326	0.72571	0.82786	0.85978	0.91250	0.84900	0.87955
(caecum and colon/wt.)	0.10094	0.14582	0.12162	0.16889	0.23351	0.25353	0.24874	0.26268
(small intestine/wt.)	0.51856	0.58846	0.52276	0.55995	0.57337	0.63103	0.62090	0.64156
(liver/wt.)	0.71518	0.70078	0.71743	0.76112	0.71438	0.68543	0.69608	0.73150
(heart/wt.)	1.47006	1.56051	1.54135	1.55794	1.51505	1.51684	1.54698	1.58597
(thyroid/wt.)	1.00091	1.08658	1.13538	1.18012	1.01206	1.06800	1.10660	1.22578

TABLE 4
ANALYSIS OF VARIANCE OF LOGARITHMS OF MEASUREMENTS AND RATIOS AT 70 DAYS

Source of Variation	Body Weight		Body Length		Body Width		Unwashed G.I. Tract		Stomach	
	M	F	M	F	M	F	M	F	M	F
Ration	.15988**	—	.00694**	—	.03454**	.00886**	.03668*	—	.06014**	.19917**
Selection	.14191**	—	.00679**	—	.01674**	.02830**	.11326**	—	.17290**	.07973**
Interaction	.00004	.03539**	.00001	.00176**	.00036	.00090	.03521	.15559**	.00010	.00096
Error	.00333	.00417	.00028	.00032	.00135	.00089	.00929	.01012	.00303	.01071

Source of Variation	Caecum and Colon		Small Intestine		Liver		Heart		Thyroid	
	M	F	M	F	M	F	M	F	M	F
Ration	.07460**	—	.00057	—	.20190**	.02465	.13851*	—	.20712**	—
Selection	.07978**	—	.06161**	—	.16787**	.03511*	.04562	—	.04523*	—
Interaction	.00006	.02411*	.01397	.09715**	.00150	.00963	.00006	.05556**	.00788	.13183**
Error	.00366	.00407	.00410	.00478	.00542	.00872	.03161	.00457	.01032	.01017

* Significant at 5% level of probability.
** Significant at 1% level of probability.

TABLE 4 (*Continued*)

Source of Variation	Body Length/ Weight		Body width/ Weight		G.I. Tract/ Weight		Stomach/ Weight		Caecum and colon/ Weight	
	M	F	M	F	M	F	M	F	M	F
Ration	.10019*	.01222	.01094	—	.48906**	.31495**	.42282**	—	.45354**	.27639**
Selection	.08659*	.08155**	.22397	—	.02396	.03681	.00262	—	.00880	.00800
Interaction	.00005	.01007	.01858	.10549*	.00517	.01628	.00011	.02245**	.00280	.00132
Error	.00204	.00381	.02240	.02004	.01249	.01014	.00344	.00325	.00183	.00221

Source of Variation	Small Intestine/ Weight		Liver/ Weight		Heart/ Weight		Thyroid/ Weight	
	M	F	M	F	M	F	M	F
Ration	—	.10283**	.00300	.01348	.00697	.00416	.00227	—
Selection	—	.00377	.00114	.08113	.00816	.00638	.35861**	—
Interaction	.01236*	.00301	.00284	.00138	.00013	.00067	.01072	.02797*
Error	.00226	.01320	.00152	.00483	.01912	.00988	.00676	.00680

* Significant at 5% level of probability.
** Significant at 1% level of probability.

There was evidence of genetic and environmental destabilization for some but not all of the traits. There was also evidence of genetic-environmental interaction, but it was more frequent among the females. Selection had an effect on over-all body proportions while ration influenced the relative size of the digestive trait.

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MORPHOGENETIC STUDIES OF THE RABBIT. XXVII. THE POSTNATAL GROWTH IN WEIGHT AND THICKNESS OF THE VENTRICULAR WALLS OF THE HEART

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Received March 14, 1960

Unlike the size of the entire heart, which has been studied for all periods of life in man and in many other animals, the weight and thickness of the two ventricular walls have received but scant attention. One of the earlier studies of the weights of the ventricular walls in man is that of Müller (1883). Some of the subsequent studies have been reviewed in a report on the weight and thickness of the ventricular walls in two inbred races of adult rabbit hearts (Latimer and Sawin, 1959).

The changes in the relative size of the entire human heart during postnatal life are reviewed by Scammon (1927), Dragendorff (1938) and Krogman (1941), and a few data are included on the changes in the weight and thickness of the ventricular walls. Due to the variability of the material, both inherent and caused by the different methods of separating the two ventricular walls from the remainder of the heart, there is need for additional quantitative studies of the human and animal hearts. The time of attainment of the adult proportions of the weight and thickness of the two ventricular walls needs further study.

MATERIALS AND METHODS

The hearts used in this study were taken from a miscellaneous group of 812 rabbits raised at the R. B. Jackson Memorial Labora-

[1] This investigation was supported (in part) by PHS research grant C281C from the National Cancer Institute, Public Health Service, and aided by a grant from the American Cancer Society.

tory. The hearts had been preserved in formalin from one to several years before the dissections were made. Since body weight, age, sex and race is known for only some of these, the total heart weight is used as the basis for comparing the weight and thickness of the ventricular walls. Other abscissae were tried, but the most uniform results were obtained by the use of the total heart weight. These rabbit hearts vary from newborn to adult and the total heart weights range from 0.5 to 13.0 gm.

All of these 812 hearts were dissected by the same person, using the same methods employed in the study of the hearts previously reported (Latimer and Sawin, 1959) and these details need not be repeated here. These ventricular walls were firm due to the preservation and furthermore there was a difference in the condition of the heart musculature at the time of preservation, some were in systole and some in diastole with the resulting differences in the thickness of the ventricular walls.

The weight of each ventricular wall as a percentage of the respective total heart weight was found. The ratios of the weight and the thickness of the two ventricular walls for each individual heart were also determined and all of these data, including weights in grams and thickness in millimeters of the individual ventricular walls, were plotted against total heart weight.

The weight in grams and the thickness in millimeters gave evidence of progressive changes with increase in total heart weight and regression formulae were determined by the method of least squares for these measurements. The weights of the walls as percentages of total heart weight and the ratios of both weight and thickness evidenced no progressive changes. They were rather uniformly scattered throughout the entire range of heart weights, or from 0.5 to 13.0 gm., and these data are treated statistically.

Weights of the Ventricular Walls

The weights of both ventricular walls plotted on heart weight increase as arithmetical functions of total heart weight throughout the entire series. The formula, developed by the method of least squares, which will predict average weights of the right ventricular wall for total heart weights ranging from 0.5 to 13.0 gm. is:

$$Y = 0.164X + 0.075,$$

and the formula for the weight of the left ventricular wall, and within the same limits of heart weights, is:

$$Y = 0.363X + 0.035.$$

In all of the formulae for weights of the ventricular walls, Y represents the weight of either the right or left ventricular wall in grams and X always represents the total heart weight in grams.

To measure the variation in these data for each regression line, the sigma, or the standard error of estimate, was determined. For the right ventricular wall this is 0.380 and for the left wall, 0.827. The preliminary graphs, or scatter diagrams of the weights of both ventricular walls when plotted on heart weight, show surprisingly little dispersion; both series of weights were grouped rather closely around the regression lines.

The sex of some of these hearts was known and there were 236 male and 376 female hearts. The weights of the ventricular walls in the male and in the female rabbit hearts were plotted and regression formulae, similarly determined by the method of least squares, were derived. The formula for the weight of the right ventricular wall for the males is:

$$Y = 0.189X - 0.013, \text{ from 0.5 to 9.0 grams of heart weight,}$$

and for the females:

$$Y = 0.171X + 0.030, \text{ from 0.5 to 13.0 grams.}$$

The formula for the weight of the left ventricular wall is, for the males:

$$Y = 0.354X + 0.042$$

and for the females,

$$Y = 0.373X - 0.002.$$

The formula for the ventricles of the males is valid only to 9 grams of heart weight while that for the females is 13.0 grams. There were only three male hearts weighing more than 9 grams. This is not surprising since the female rabbits generally weigh more than the males.

No attempt was made to group these hearts by races for many were not identified as to their race, and thus there were insufficient numbers for the development of regression formulae.

Thickness of the Ventricular Walls

The thicknesses of the ventricular walls are much more variable than the weights in grams, and unlike the weights, these measurements do not increase in one continuous series. The increase in thickness of both ventricles is more rapid in the smaller, and slower in the larger hearts. Furthermore the formulae for thickness of both walls do not fit the 28 cases between 0.5 and 1.0 gm., for these are evidently increasing in thickness very rapidly.

The thickness in millimeters of the right ventricular wall in hearts weighing from 1.0 to 6.5 gm. may be estimated from the formulae:

$$Y = 0.103X + 0.762, \text{ and from 6.5 gm. to 13.0 gm.,}$$
$$Y = 0.089X + 0.854.$$

The formula for the thickness of the left ventricular wall from 1.0 to 4.7 gm. is:

$$Y = 0.558X + 1.645; \text{ and from 4.7 to 13.0 gm.,}$$
$$Y = 0.171X + 3.479.$$

In all of these formulae for thickness, Y represents the thickness in millimeters and X, the total heart weight in grams. The sigma or standard error of estimate for the thickness of the right ventricular wall is 0.290 and for the left, 1.054 or three times that for the right.

This greater variability in thickness of the ventricular walls may be due in part to the difficulty of accurately measuring a very irregular wall, and in part to the variable distension of the ventricular walls by the varying amount of contained blood.

Since there is considerable variability in the thickness of these walls, correlation coefficients between the weight and thickness of each wall were calculated to determine the degree of similarity in the development of their weight and thickness. The correlation coefficient between the weight and thickness of the right ventricular wall is + 0.600, and for the left, + 0.733. Thus the increases in both thickness and in weight of the two ventricular walls are very closely related throughout this series of rabbit hearts.

Percentage Weights

The weight of each ventricular wall of each of the 812 rabbits was reduced to a percentage of the respective total heart weight and these percentages plotted against heart weight as were the weights in grams.

Unlike the weights, these percentages exhibited no significant changes through the entire range of heart weights. They were scattered, but in neither field graph were there any significant trends. These percentage weights were studied statistically and the usual statistical constants are shown in the first two columns of panel A, Table 1.

TABLE 1

AVERAGES, STANDARD DEVIATIONS, AND COEFFICIENTS OF VARIATION FOR THE PERCENTAGE WEIGHTS AND THE RATIOS OF WEIGHT AND THICKNESS OF THE RIGHT AND LEFT VENTRICULAR WALLS OF THE RABBIT HEART

	Right wall % heart	Left wall % heart	Ratio LVW/RVW grams	Ratio LVW/RVW mm
	Panel A all 812 hearts together			
Average	18.10	37.11	2.09	3.20
Standard deviation	2.41	3.92	0.391	0.60
Coef. variation	13.34	10.57	18.67	18.83
	Panel B 236 males			
Average	18.38	36.71	2.05	3.11
Standard deviation	2.18	4.25	0.390	0.557
Coef. variation	11.89	11.58	19.04	17.88
	Panel C 376 females			
Average	17.94	37.12	2.11	3.36
Standard deviation	2.34	3.86	0.387	0.661
Coef. variation	13.04	10.40	18.36	19.70
"t" values of percentages	2.32 (5%)	1.23	1.92	4.67 (1%)

The percentage weights of the two ventricular walls of the 236 male and 376 female rabbits are shown in the first two columns of panels B and C of Table 1. The last line of panel C shows the "t" values of the differences in these male and female percentages. The percentage of the right ventricular wall is significantly larger (5%) in the male rabbits and the left is slightly, but not significantly larger in the females. The percentage weight of the right wall is more variable in the females and the left is more variable in the males.

RATIOS OF WEIGHT AND THICKNESS

The ratios between the weight in grams and the thickness in millimeters for both ventricular walls were found for each rabbit. These ratios, like the percentage weights, gave no evidence of significant trends when plotted on total heart weight, except for the ratios of thickness in the 28 rabbit hearts below one gram in weight, as dis-

cussed below. These ratios for both weight and thickness for all 812 rabbits are shown in the last two columns of panel A, Table 1. Similar ratios for the males are shown in panel B, and for the females in panel C of Table 1. The ratios of both weight and thickness of the two ventricular walls are greater in the females (last line panel C). The ratios of thickness are significantly greater while those of the weights are larger but not significantly larger.

All of the rabbit hearts under two grams in weight were separated into groups of 0.5 gm. range and the ratios averaged for these three groups. The ratios of the weights of the two ventricular walls evidence no significant differences between these groups nor from the total average of all of the hearts. Hence we may conclude that by the time the rabbit heart has reached a weight of 0.5 gm. the relative weight of the two ventricles have attained their adult proportions, unlike the similar proportions of the ventricular walls in the human infant (Scammon, 1927).

The ratios of the thickness of the two walls in these three groups of smaller hearts, however, are not all alike. The ratio of thickness in the group from 0.5 to 1.0 gm. is only 2.74 which is significantly less than for the two succeeding groups and for the general average. Thus the smaller hearts under one gram in weight show a difference in the ratios of the thickness of the walls while there is no appreciable difference in the ratios of their weights.

Discussion

Since the percentage weights and the ratios of both weight and thickness show no progressive changes throughout the postnatal growth period they are compared in Table 2 with similar data from two

TABLE 2
Comparisons of 812 Miscellaneous Rabbit Hearts with Hearts of Adult Rabbits of Dwarf Race X, Normal Race X, Race III and Hearts of Adult Dogs

	Misc.	Dwarf X	Normal X	III	Dogs
R.V.W. (gm.) % heart	18.10	17.80	17.94	21.80	22.02
Coef. var.	13.34	9.26	12.10	6.91	7.47
L.V.W. (gm.) % heart	37.11	37.26	36.24	34.79	39.48
Coef. var.	10.57	10.42	9.16	6.05	6.49
Ratio (gm.) LVW/RVW	2.09	2.10	2.04	1.60	1.82
Coef. var.	18.67	12.09	14.67	9.46	11.23
Ratio thickness	3.20	2.95	3.11	2.48	—
Coef. var.	18.83	14.80	17.84	14.21	—

inbred races of rabbits (Latimer and Sawin, 1959) and with a series of adult dog hearts (Latimer, 1959). Race X was composed of both normal and heterozygous dwarf rabbits (Latimer and Sawin, 1955) and the data for these are shown separately in columns two and three of Table 2. In general the data for this present series of rabbits are more like those of race X than race III rabbits or the dogs.

The percentage weights of the right ventricular wall (line 1, Table 2) tend to parallel the body weight for they are smallest in the dwarf rabbits, larger in race III, and still larger in the dog hearts. The percentage weights of the left ventricles, however, do not follow the same pattern, for the smallest percentage is found in race III and the largest in the dogs. It was suggested in the previous publication (Latimer and Sawin, 1959) that the percentage weight of the left ventricular wall parallels the degree of activity of the animal. This holds true for the animals represented in Table 2 if we concede that the dogs are more active than any of these rabbits. Race X rabbits are certainly more active than race III.

Table 3 of the earlier report (Latimer and Sawin, 1959) and data from several authors quoted by Tandler (1913) and Dragendorff (1938) and measurements made by Truex and Warshaw (1942) show a rather wide range in the ratios of both weight and thickness of the two ventricular walls. This probably is due in part to the inherent variability of the material; in part to the various methods of separating and of measuring the ventricular walls, and also possibly to the various species of animal hearts studied. More data carefully and uniformly prepared and also for more species of animals are needed to clarify many of these points.

Dragendorff (1938) quotes figures from Müller (1883) showing that the ventricles of the human heart are relatively smaller compared with the weights of the atria until about the end of the first year of postnatal life, at which time these two parts assume their adult quantitative relationships. The weights of the atria were not determined, but the two ventricular walls in the 28 smallest rabbit hearts, less than 1 gm. in weight, of this present series, form 55.7% of the total heart weight and for the entire 812 rabbits, the percentage is 55.2%. Thus the relationship of the ventricles to the entire heart weight appears to be fairly well established in these 28 smallest hearts of this series of rabbits.

A ratio of 1.96 for the weights of the two ventricular walls in 64 adult male guinea pigs (Latimer, 1952) is not very different from the ratios for the rabbit and dog hearts in Table 2.

The thickness of the two ventricular walls for 500 human cadavers, 100 cats and for 12 specimens in each of 7 other species of animals have been reported by Truex and Warshaw (1942). Ratios of the thickness of the ventricular walls have been computed from their measurements and these ratios range from 1.94 to 3.50. Their measurements of the thickness of the walls were made at the thickest part of the wall distal to the coronary sulcus, while the measurements of all of the hearts, both human and animal, studied in this laboratory were the minimum measurements possible of the anterior wall, just distal to the coronary sulcus. The right ventricle was measured along the medial axis of the heart and not in the region of the thin walled conus arteriosus. Their ratio of 3.12 for the human heart is 17% greater than the ratio of 2.66 reported by Latimer (1953). This difference of 17%, however, is not excessive when one considers the differences in the methods, and the irregularities of the left wall. The ratios calculated from their measurements are as follows: cat, 3.50; human heart, 3.12; dog, 3.10; monkey, 3.10; sheep, 2.58; calf, 2.28; beef, 2.24 and the pig, 1.94. This series suggests that these ratios differ according to the species. The position in the series of the pig and cow would lead one to infer that the activity of the animal might have something to do with the size of the ratio.

Tandler (1913) and Dragendorff (1938) review the earlier literature and list the weight and thickness of the two ventricular walls in man, and from these data the ratios of weight and thickness have been computed for comparison with the rabbit ratios. The ratios for the weights of the two ventricular walls vary from 1.85 to 2.08 or much like the ratios in line 5 of Table 2. Tandler quotes measurements of thickness from which ratios varying from 2.08 to 3.26 were computed. All of these except the lowest ratio of 2.08 which was reported by one worker (Krause) are similar to the ratios in next to the last line of table 2.

Dragendorff (1938) and Krogman (1941) list weights of the two ventricular walls for the late fetal period through adolescence. The ratios determined from these weights are somewhat conflicting, although in general they show that at about the time of birth the two

walls are more alike in weight. Then at from three to 6 months after birth (Tandler, 1913) they attain their relative adult proportions. The various data quoted do not agree upon just when the relative adult proportions are attained. In the group of 28 smallest hearts of this present series of rabbits, or those under one gram of heart weight, the ratio between the weights of the two ventricular walls is 2.11 or slightly above the average of 2.09 for the entire series. The formulae for the weights of the ventricular walls apply equally well to these smallest hearts, and the preliminary graphs showed all of these cases closely grouped around the regression lines. This is not true for the thickness of the walls, for the ratio of thickness of the two walls for the group of 28 smallest hearts is 2.74. The hearts weighing from 1.0 to 1.5 gm. have an average ratio of 3.05 which is still slightly below the average ratio for the thickness of 3.20.

Thus the ratios of the weights of the two ventricular walls in the smallest group of rabbit hearts is practically equal to the adult ratios in this series of rabbits, while the normal adult ratio of the thickness of the two walls is not attained until the hearts reach a weight of 1.5 to 2.0 gm. The regression lines for the thickness of the two ventricular walls, unlike those for the weights of the walls, show a more rapid increase in the smaller hearts than in the larger hearts. All of this seems to suggest that the two ventricular walls in these rabbits do not attain their adult ratios of thickness as soon as the adult ratios of weight. More hearts in the smallest group and also hearts of still smaller and younger rabbits are needed to confirm this observation. Furthermore, the ages of these rabbits should be known for more definite conclusions concerning the time of the attainment of the adult ratios of both weight and thickness.

Summary

This study concerns the weight and thickness of both ventricular walls of 812 rabbit hearts, ranging in weight from 0.5 to 13.0 gm. The weights of both walls as percentages of total heart weight and the ratios of the weight and the thickness of the two walls are also presented.

The weights of both ventricular walls form continuous series when plotted on total heart weight, and empirical formulae have been found by the method of least squares for estimation of the weight of right

and left ventricular walls from the weight of the entire heart.

The thickness of the two walls increases more rapidly in the smaller hearts and more slowly in the larger hearts. Empirical formulae are presented showing the changes in the thickness of each wall.

The right ventricular wall averages 18.10% and the left wall, 37.11% of the heart weight throughout the entire series.

The ratios of the weight of the left to the right wall in all of the hearts averages 2.09.

The similar ratio for the thickness of the two walls in all hearts above 1 gm. in weight averages 3.20. The ratio of the thickness in hearts less than 1 gm. in weight is 2.74. The adult ratio of weight is attained by 0.5 gm. of heart weight while the adult ratio of the thickness of the two walls is not attained until the heart has exceeded a weight of 1 gm.

These data are compared with similar measurements in two inbred races of rabbits and other mammals including man.

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Growth, 1960, **24**, 69-80

A HISTOCHEMICAL STUDY OF INSULIN IN THE CHICK EMBRYO PANCREAS[1]

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Received March 31, 1960

INTRODUCTION

The period of 10 to 14 days of incubation appears to be critical in the development of endocrine control of carbohydrate metabolism in the chick embryo (Willier, 1955). The possible role of insulin in this transitional period has been indicated by various studies. Potvin and Aron (1927) and Aron (1930) concluded that insulin was first secreted on day 12 of incubation after observing that the initial appearance of definitive pancreatic islets corresponded in time with what they considered to be the onset of liver glycogen deposition. Villamil (1942) postulated the synthesis and release of this hormone on 12 to 13 days of incubation based upon the observation that at this time chrom-alum-hematoxylin positive granules first appeared in the beta cells. However, more recent findings are in striking disagreement. Ghiani (1956) has found that paraldehyde-fuchsin (P.A.F.) positive granules first appear in the beta cells of the chick pancreas on the 14th day of incubation. On the other hand, using the same technique as Villamil, he could not demonstrate chrom-alum-hematoxylin positive granules until the 16th day. More recently, Lievre (1957a) has found that P.A.F. positive elements make their first appearance in 17-day embryos. In marked contrast, hypophysectomy by means of X-irradiation seemingly causes a precocious development of beta elements on day 10 of incubation (Lievre, 1957b).

The present study was undertaken to reevaluate the findings of the previous investigators by means of a histochemical technique for the

[1] Supported by research grant A-2718(A) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U.S.P.H.S.

[2] The author would like to express his appreciation for the technical assistance of Mrs. Betty D. Annan and Mr. Joseph Bullaro.

demonstration of insulin. Paraldehyde-fuchsin stained material was also examined.

Materials and Methods

All embryos were obtained from White Leghorn eggs incubated at 38.3 ± 1.1 degrees C. in forced-draft incubators. The ages of all specimens represent the actual time the eggs remained in the incubator.

Three series of embryos were prepared. Each group consisted of 6 embryos per day from the 6th through the 18th day of incubation. At the time of sacrifice the embryos were removed from the shell and rinsed in saline, a cut being made in the abdominal wall to allow for a more rapid penetration of fixative. Specimens were fixed either in Bouin's solution, Romeis fluid or in trichloroacetic acid in 80% alcohol (T.C.A.). Romeis fixation renders insulin insoluble within the pancreatic tissue whereas insulin is T.C.A. soluble (compare Figs. 2, 5, and 7 with Fig. 10).

After fixation, the intestinal loop containing the pancreas was removed from the embryo and the gland gently dissected from the intestine. The organ was subsequently washed in 70% alcohol, dehydrated in increasing percentages of alcohol, cleared in xylol and embedded in paraffin. Sections were cut at 6 μ and were mounted serially.

The Bouin-fixed materials were stained with Gomori's paraldehyde-fuchsin (1950) after the modification of Scott (1952) for the demonstration of beta granulation. The Romeis- and T.C.A.-fixed materials were stained by the technique of Barrnett *et al.* (1955) for the histochemical demonstration of insulin.

All photomicrographs were made with Kodak Panatomic-X film and printed on high contrast paper.

Observations

The differentiation of the endocrine portion of the developing chick pancreas begins on the 7th or 8th day of incubation (Potvin and Aron, 1927; Aron, 1930; Villamil, 1942; Ghiani, 1956; Lievre, 1957a, 1957b). However, the exact time at which beta granules first appear within the islet tissue is undecided (Villamil, 1942; Ghiani, 1956; Lievre, 1957a, 1957b).

In the present series of investigations, histochemically demonstrable insulin, as well as P.A.F. positive granules, are first apparent in the

pancreatic tissue on the 7th day of incubation. In pancreases of 7-day chick embryos, beta cells are sparsely distributed throughout the relatively undifferentiated cellular components of the pancreas (Fig. 1).

On days 8 and 9 of incubation the differentiation of beta elements becomes more striking as evidenced by both D.D.D. and P.A.F. (Figs. 2 & 3).

In 10-day chick embryos the separation of beta and acinar cells becomes quite distinct (Figs. 4 & 5). It is at this time that an unequivocal equation of D.D.D. positive cells and P.A.F. positive cells can be made on the basis of position and distribution alone.

Beginning on the 11th day (Figs. 5 & 6), and extending through the 13th day of incubation (Figs. 7 & 8), there is a marked increase in reactivity of the beta cells to both stains. Not only does the intensity of the D.D.D. reaction increase, but the numbers of P.A.F. positive granulations are greatly elevated. In addition, the exocrine tissue which up to this time has shown only a slight affinity for D.D.D. and P.A.F. now stains considerably, indicating a distinct rise in sulfhydryl content and the onset of zymogen granulation production.

In the glands of 14- through 18-day chick embryos the number of beta cells and beta granules as well as histochemically demonstrable insulin remains relatively constant (Figs. 9, 11, 12).

There is a progressive increase in the density of acinar zymogen granules from the time of their initial appearance until the 16th day, after which time there is apparently no quantitative change.

Discussion

The appearance of histochemically demonstrable insulin as well as histologically demonstrable beta granulations within the islet tissue of the pancreas of 7- to 8-day chick embryos is in striking contrast with the findings of previous investigators (Villamil, 1942; Ghiani, 1956; Lievre, 1957a). The differences in reported time of appearance of beta granulations (insulin or a precursor) are most probably the result of differences in technique and/or interpretation of results. Both Villamil (1942) and Ghiani (1956) used Gomori's chrom-alum-hematoxylin technique but found beginning beta granulation on the 12th and on the 16th days of incubation, respectively. The variability in technique and in interpretation is also apparent when the supposedly more specific paraldehyde-fuchsin method is employed. Ghiani (1956)

PLATE I

FIGURE 1

Cross section of the pancreas of a 7-day chick embryo. P.A.F. positive cells scattered through relatively undifferentiated pancreatic tissue. Final magnification 1300X.

FIGURE 2

Cross section of the pancreas of an 8-day chick embryo. D.D.D. positive cells found among the differentiating acinar cells. Final magnification 1300X.

FIGURE 3

Cross section of the pancreas of a 9-day chick embryo. P.A.F. positive cells. Final magnification 1300X.

FIGURE 4

Cross section of the pancreas of a 10-day chick embryo. P.A.F. positive cells. Final magnification 1300X.

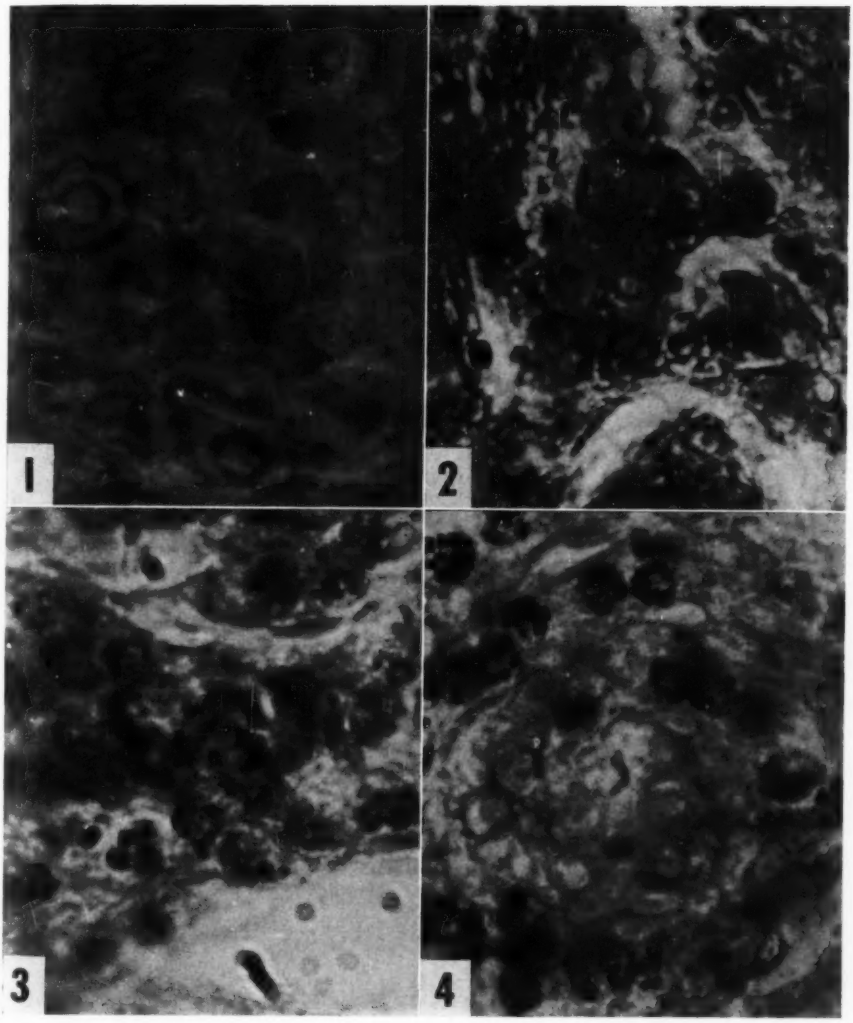
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PLATE II

FIGURE 5

Cross section of the pancreas of an 11-day chick embryo. D.D.D. positive islet cells. Final magnification 1300X.

FIGURE 6

Cross section of the pancreas of an 11-day chick embryo. P.A.F. positive cells. Final magnification 1300X.

FIGURE 7

Cross section of the pancreas of a 13-day chick embryo. D.D.D. positive cells distributed in strands between masses of acinar tissue. Final magnification 1300X.

FIGURE 8

Cross section of the pancreas of a 13-day chick embryo. P.A.F. positive cells. Final magnification 1300X.

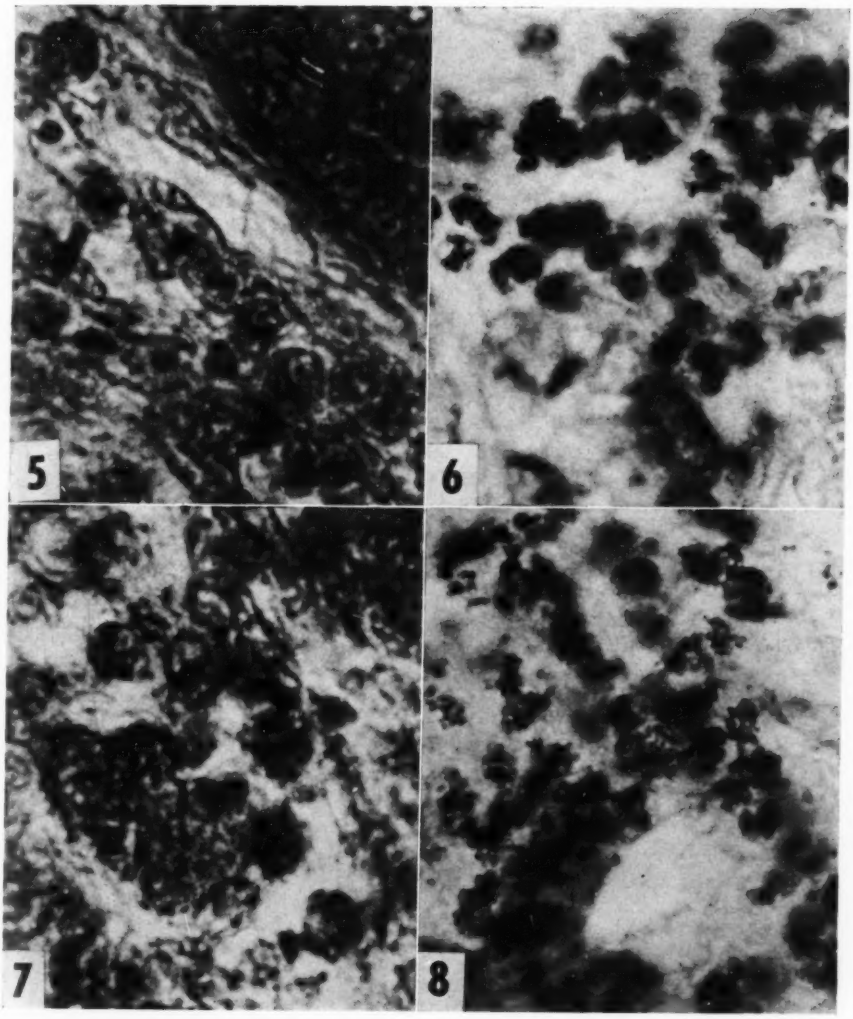
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PLATE III

FIGURE 9

Cross section of the pancreas of a 15-day chick embryo. P.A.F. positive cells. Final magnification 1300X.

FIGURE 10

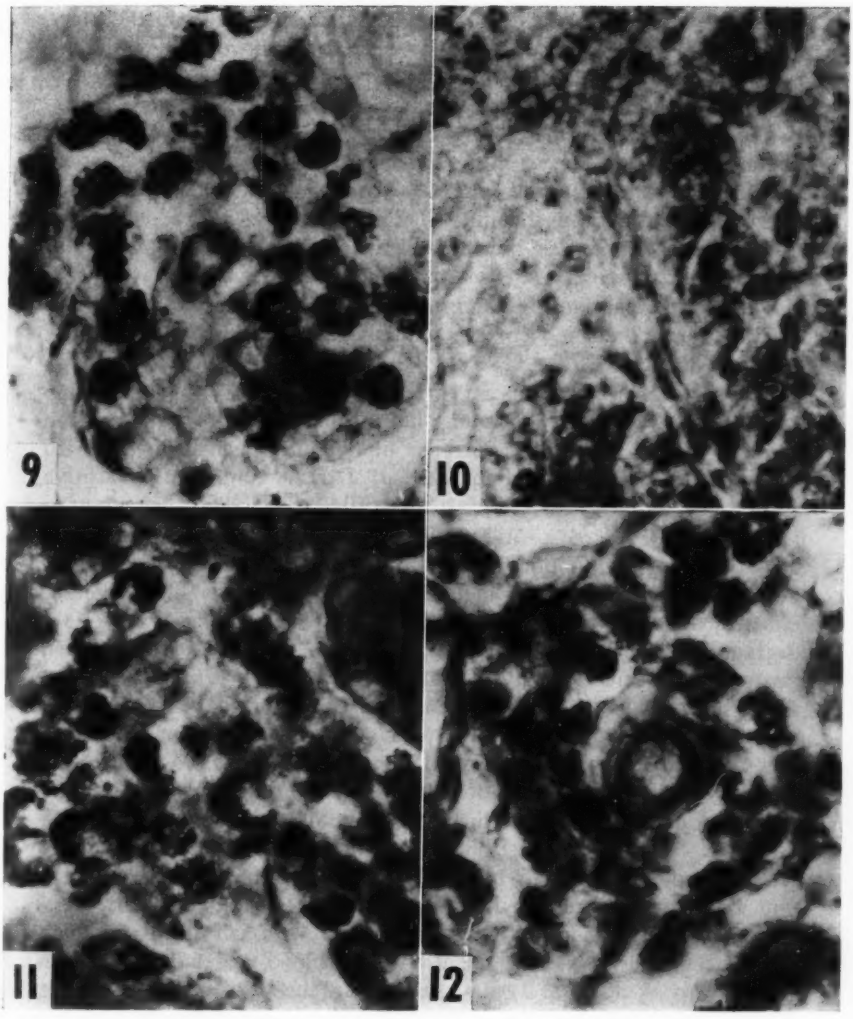
Cross section of the pancreas of a 15-day chick embryo. D.D.D. reaction of islet tissue after removal of insulin by T.C.A. fixation. Final magnification 1300X.

FIGURE 11

Cross section of the pancreas of a 17-day chick embryo. P.A.F. positive cells distributed in strands throughout the highly reactive acinar tissue. Final magnification 1300X.

FIGURE 12

Cross section of the pancreas of a 18-day chick embryo. P.A.F. positive cells distributed around pancreatic duct. Final magnification 1300X.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using the P.A.F. technique describes the appearance of beta elements (granulations) on day 14 of incubation; Lievre (1957a), on the other hand, maintains that P.A.F. positive granules first appear within the islet tissue on day 17. However, in a study of the effects of hypophysectomy upon development of beta granulation within the pancreas, this investigator describes the appearance of P.A.F. positive elements (beta granules) in 50% of 10-day control embryos. In the present series of investigations, the consistency of reaction both with 2, 2′-dihydroxy-6, 6′-dinapthyl disulfide (D.D.D.) and P.A.F. adds credence to the above data which indicate that the initial synthesis of insulin occurs in the pancreas of 7- to 8-day chick embryos.

The early appearance and subsequent increase of insulin within the chick embryo pancreas corresponds in time with two major ontogenetic events.

Liver glycogen appears as early as the 6th day of incubation (Jenkins, 1955) with a gradual increase in amount from the 7th through the 9th day. This increase of liver glycogen coincides with the initial appearance and increase of D.D.D. and P.A.F. positive substances within the developing beta cells.

The striking increase in D.D.D. and P.A.F. elements during the 11- to 14-day incubation period occurs during the aforementioned "transitional period" in the endocrine control of carbohydrate metabolism in the chick embryo. During this same time interval there is a decline in the amount of liver glycogen on the 12th day with a marked "recovery" on the 13th day of incubation (Jenkins, 1955). Similarly, blood sugar levels rise from the 8th through the 10th day. These levels remain constant until the 14th day when there is a pronounced rise in blood sugar (Leibson & Leibson, 1943; Konigsberg, 1954). Injection of insulin into the yolk sac of normal chick embryos on the 5th day produces, during the succeeding days, diminution of all carbohydrate fractions (free sugar, glycogen and bound carbohydrate) within the embryo proper. However, by the 12th day the amounts of free sugar and glycogen have returned to normal levels (Zwilling, 1951). Also, injection of adrenocortical extract into the yolk sac of 5-day old chick embryos produces a subsequent rise in blood sugar levels which reaches a peak at 10 to 12 days, returning abruptly to normal values by the 14th day (Zwilling, 1948). Konigsberg (1954) has found a marked, increasing hyperglycemia characteristic of the blood of surgi-

cally hypophysectomized chicken embryos from the 8th (the first day studied) to the 12th day of incubation. In succeeding days there is a decided trend downward until normal levels are reached by the 16th day. Likewise, Watterson *et al.* (1958) have shown that hypophysectomy markedly lowers the rate of glycogen accumulation by the glycogen body beginning on the 14th day. However, Snedecor and Henrikson (1959) have found that administration of insulin to chick embryos failed to have any effect on the glycogen content of the glycogen body.

Thus, the time of appearance of histologically and histochemically demonstrable insulin corresponds approximately with the time of initial glycogen deposition and its subsequent increase within the liver. The decided increase of D.D.D. and P.A.F. demonstrable materials between 11 and 14 days of incubation seems to suggest that the pancreas is partially responsible for the changes in carbohydrate metabolism which occur during this period.

Summary

1. A histochemical and histological study of the chick embryo pancreas was undertaken in order to determine the time of initial synthesis of insulin and to reinvestigate the time of first appearance of beta cell granulations.

2. Histochemically demonstrable insulin and histologically demonstrable beta granulations appear first on the 7th day of incubation concomitant, in this series, with the beginnings of acinar and islet differentiation. The numbers of beta cells and beta granulations, as well as the amount of histochemically demonstrable material, are initially sparse.

3. In 8-, 9-, and 10-day chick embryo pancreases there is a gradual and steady increase in all of these elements.

4. On the 11th through the 13th day of incubation there is a striking increase in histochemically demonstrable material and in the amount of beta granulation.

5. These tinctorial elements remain rather constant in amount from the 14th through the 18th day of incubation.

6. Zymogen granules in the acinar tissue appear during the 11th through the 13th day and increase in amount up to the 16th day, remaining relatively constant through the remaining embryonic period.

7. The early appearance of histochemically demonstrable insulin and beta granulation and their subsequent increase has been correlated with known physiological events within the chick embryo.

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Growth, 1960, **24**, 81-82

BOOK REVIEW

BLAKESLEE: THE GENUS DATURA. Amos G. Avery, Sophie Satina, Jacob Rietsema. Foreword and Biographical Sketch by Edmund W. Sinnott. xii + 289 pp., 67 illustrations. No. 20 of Chronica Botanica, an International Biological and Agricultural Series. New York (The Ronald Press Co.) 1959. $8.75.

When Albert Francis Blakeslee (1874-1954) retired in 1942 from the post of Director of the Cold Spring Harbor Station of the Carnegie Institute of Washington, he was offered a professorship at Smith College. Here he established a Genetics Experiment Station, which he directed until he died in his eightieth year. The authors of this memorial to Blakeslee and his work were all connected with the Smith College Station.

As a graduate student, Blakeslee acquired the habit of making important discoveries in any field in which he really became interested. His work on the Mucorales, where he discovered heterothallism and the beginning of sex, was the occasion of his first major contribution; but he is best known today for the many papers he published on the genus *Datura*.

The present volume consists of fifteen review papers written chiefly by the three authors in various permutations and combinations. In addition, there is included a paper on the "Alkaloids of Datura," by Edward Leete. Two junior authors also, Jean M. Cummings and Benigna Blondel, have each contributed to a paper. These papers taken together bring the important *Datura* story up to date.

The book is introduced by an explanatory foreword by Edmund W. Sinnott, who has also contributed a brief but very complete Biographical Sketch of Blakeslee. A *Datura* bibliography of 228 items that lists the papers of Blakeslee and his colleagues, arranged in sequence from 1910 to 1956, adds greatly to the value of the book as a handy reference source. Every botanical and genetics library will, sooner or later, have to get a copy.

The genetic investigation of the genus *Datura*, as carried on by Blakeslee and his collaborators turned out to be exceptionally profitable, and *Datura* now ranks with *Zea*, *Nicotiana*, *Oenothera*, *Crepis* and *Pisum* as a genus in which major discoveries in plant genetics have been made. *Blakeslee: The Genus Datura*, records and system-

atizes these discoveries and, in addition, places them in the overall setting of our knowledge of the genus. The several papers cover a historical review, a taxonomic history, the *Datura* alkaloids, the chromosome numbers and morphology, polyploidy, the types produced by extra chromosomes, gene mutations, chimeras, radiation experiments, life history including description of the gametophytes and sporophytes, growth processes in embryo and seed and the seed development, segmental interchanges and their bearing on the species problem and, finally, the various barriers to crossability. The papers are necessarily technical but they are very clearly written. All in all the book is an appropriate memorial to a man well loved and universally respected.

CONWAY ZIRKLE

February 15, 1960